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(54) Title: EFFICIENT *EX VIVO* EXPANSION OF CD4+ AND CD8+ T-CELLS FROM HIV INFECTED SUBJECTS

(57) **Abstract**

Methods for the expansion of CD4, CD8, and DP T-cells from HIV infected patients are disclosed which allow the maintenance of low levels of HIV. The invention further discloses methods for the inhibition of HIV gene expression. Also disclosed are methods for the rapid and efficient screening of cells derived from HIV-infected patients to assess the suitability of various antiviral treatments. The invention further provides a means for the generation of cell banks for use in immune reconstitution and means of treating or modifying expanded cell populations prior to infusion to enhance or modulate therapeutic effectiveness.

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TITLE OF THE INVENTION

EFFICIENT *EX VIVO* EXPANSION OF $CD4^+$ AND $CD8^+$ T-CELLS FROM HIV INFECTED SUBJECTS

BACKGROUND OF THE INVENTION

The human immunodeficiency virus (HIV) is a serious and growing health threat in virtually every part of the world. It has been estimated that over 22 million people are currently infected worldwide, and it is anticipated that over 40 million people will be infected by the end of this decade.

HIV infection typically leads to acquired immunodeficiency syndrome (AIDS) within 8 to 10 years after infection. Individuals with AIDS are subject to opportunistic infections and cancers, leading to severe illness and, ultimately, death. Although various treatments delaying the progression from HIV infection to AIDS are known, these treatments are of limited effectiveness and generally require the use of pharmaceuticals which have adverse side-effects. Moreover, the effectiveness of different drug treatments varies among individuals and no satisfactory system exists to screen different drug combinations for effectiveness in combating HIV infection in a particular subject.

HIV is a retrovirus, closely related to simian immunodeficiency virus (SIV). At least three variants of HIV, known as HIV-1, HIV-2, and HIV-0, are known. It is believed that HIV-1 is the predominant global form of human HIV infection at the present time. HIV-2 is believed to be common in West Africa, but rarer elsewhere in the world. HIV-2 appears to be less pathogenic that HIV-1. In addition to these three HIV variants, the high natural mutation rate

of HIV DNA means that virtually every individual infected with HIV carries a slightly different virus. Differences between HIV isolates complicate efforts to devise effective anti-HIV approaches, including drugs and vaccines.

HIV enters target cells by the binding of gp120 (present on the HIV virion) to cellular receptors, followed by fusion of the viral envelope with the plasma membrane of the target cell. The major cellular receptor for the HIV gp120 is cluster of differentiation factor 4 (CD4). The highest levels of CD4 are generally found on T-helper (Th) cells; thus, the consequences of HIV infection are typically most obvious in the Th cell population. HIV can also infect other cells, including macrophages, monocytes, dendritic cells, Langerhans cells, and microglial cells. HIV-1 has a higher affinity for CD4 than does HIV-2, and it is thought that this may contribute to the greater pathogenicity of HIV-1 compared to HIV-2. HIV-1 also requires a chemokine coreceptor (e.g. CCR5 or CXCR4) to gain entry into susceptible cells.

There is evidence in the prior art suggesting that specific chemokines such as RANTES, MIP-1α and MIP-1β may inhibit fusion between the HIV-1 virion and target cells by inhibiting the interaction between HIV surface proteins and cell surface receptors. This inhibits viral replication by reducing the rate of infection.

Fusion between the HIV virion and the plasma membrane of the target cell allows the HIV RNA to enter the target cell, where it is reverse transcribed into DNA by viral reverse transcriptase and integrated into the host cell's genome to form an HIV provirus. Once the

HIV DNA is integrated into the host cell genome, it is replicated during cell division and is passed on to daughter cells.

The HIV provirus may remain inactive in the host cell for some time until it is activated. Upon activation, HIV structural genes are expressed, and single stranded HIV RNA (HIV ssRNA) is transcribed. The HIV structural proteins and HIV ssRNA assemble to form numerous virus particles which then exits from the host cell infects other cells.

At present, methods of inhibiting HIV replication in tissue samples have tended to focus on reducing the number of newly infected cells through the inhibition of infection by released virus particles. This has been effected through the use of compounds which inhibit fusion between the HIV virion and the plasma membrane, and inhibitors of viral reverse transcriptase (necessary to generate DNA from the viral RNA prior to integration into the host cell genome) to form the provirus. In addition, the production and release of viral particles from infected cells has been inhibited through the use of protease inhibitors which interfere with the post-translational processing of HIV gene products necessary for virus particle formation. The effectiveness of many current therapies is limited by the capacity of the HIV virus to mutate, resulting in the development of resistance. Methods for inhibiting the expression of HIV DNA in populations comprising CD8 and CD4 cells from infected subjects (thereby reducing the number of virus particles which can be formed) while greatly expanding CD4 and CD8 cells in these populations are not known in the art. Such methods might be less susceptible to circumvention by acquired resistance and therefore represent a potentially powerful form of HIV treatment.

It is desirable to have a means of inhibiting the expression of HIV DNA in infected cells. Individual infected cells are capable of producing a massive number of infectious HIV particles, and the release of such particles from a cell can cause the infection of numerous previously uninfected cells. Levine *et al.* (*Science*, 272:1939, 28 June 1996) have reported that the interaction of CD4 cells with immobilized (but not soluble) CD28 monoclonal antibodies reduces the susceptibility of CD4 cells to HIV infection. However, it would be more efficient to inhibit the formation of HIV particles in infected cells, rather than to simply attempt to reduce the rate of infection by such particles following their formation and release from the infected cell.

HIV DNA sequences are flanked by long terminal repeats (LTRs). Promoter and enhancer sequences are located in the 5' LTR, and polyadenylation sequences are contained in the 3' LTR. The 5' LTR sequence normally has only a low affinity for RNA polymerase, causing premature truncation of transcription products and preventing the formation of infectious viral particles. However, the viral protein Tat is capable of interacting specifically with a region (TAR) on the emerging RNA transcript and increasing the formation of full-length proviral transcripts.

One of the characteristic features of HIV infection is a reduction in the number of $CD4^+$ T-cells ("CD4 cells") in the peripheral blood of infected subjects. Healthy uninfected individuals typically have approximately 1 100 CD4 cells per microliter of whole blood. After an individual has been infected with HIV, CD4 cell levels generally drop gradually over

a period of 8 to 10 years, but then drops more rapidly. In subjects with AIDS, CD4 cells levels below 200 cells per μl are common.

It is believed that in the early stages of HIV infection CD4 cells are destroyed at a high rate. However, at this stage the subject's immune system is able to replace many of the destroyed cells, resulting in only a gradual decline in observed CD4 cell numbers. Cells may be destroyed by various means following infection. One means for the destruction of infected cells is lysis resulting from the exit of large numbers of newly formed virus particles. A second means by which infected cells may be destroyed is by an immune response to HIV antigens expressed on the cell membrane. It is believed that enhanced levels of active anti-HIV specific CD4 cells in HIV infected patients allows the maintenance of low viral loads and non-progression into AIDS.

It appears that many uninfected CD4 cells lose their capacity to respond to foreign antigens and are also destroyed during HIV infection. The exact mechanism by which this occurs is not fully understood. However, it is suspected that free gp120 to CD4 molecules on the surface of uninfected cells. This binding may lead to the internalization of the gp120 by the uninfected CD4 cells. Proteolytic processing of the internalized gp120 in the endosome, followed by association of the processed peptides with class II MHC, may lead to the expression of an HIV peptide-MHC complex at the surface of uninfected cells. Such cells may thus be destroyed as a result of an immune response directed at the peptide-MHC complex. Additionally, the binding of free gp120 to CD4 molecules on uninfected cells may interfere with the ability of these CD4 molecules to interact with class II MHC molecules on

antigen-presenting cells, thereby reducing the ability of the uninfected cell to participate in an immune response to foreign antigen. Alternately, the binding of gp120 to CD4 molecules on an uninfected CD4 cell may stimulate the production of an inappropriate activation signal, which may lead to apoptosis. It has also been postulated the free gp120 may bind to CD4 molecules on developing thymocytes, interfering with normal T cell maturation processes. Additionally, there is evidence suggesting that a single CD4 cell infected with HIV can fuse with large numbers of uninfected CD4 cells, forming a syncytium. Syncytia appear capable of producing large numbers of viral particles over a short period of time before they die.

There is evidence indicating that CD4 cell populations from subjects with AIDS have a significantly reduced ability to proliferate in response to specific antigens. This selective loss of responsiveness has been hypothesized to be the result of an inappropriate activating signal received by CD4 cells, leading to cellular anergy or apoptosis.

A shift in cytokine production by CD4 cells has been observed during the progression toward AIDS. As the disease progresses, the production of the Th1-type cytokines IL-2 and IFN-γ decreases and the production of the Th2 type cytokine IL-10 (and for a limited time IL-4) increases. This may reflect a shift from a Th1-type cellular immune response to a Th2-type humoral immune response. The reduction in IL-2 levels observed following HIV infection appears to impair the ability of CD8 cells to form cytotoxic T-lymphocytes, reducing the subject's ability to eliminate virus-infected cells and tumour cells. IFNγ has been reported to induce an anti-viral state in cells, and reduced IFNγ levels following HIV infection may

undermine this mechanism of cellular defense. Furthermore, IL-2 and IFNγ activate natural killer cells ("NK cells") which are important in the very early stages of viral infection.

The cellular depletion observed following HIV infection appears to primarily affect the CD4 cell population. However, the infection and eventual loss of dendritic cells may play an important role in disease progression. Dendritic cells are major antigen presenting cells and are important to T cell activation. Dendritic cells are also important in the maintenance of functional lymph nodes, wherein T cell and B cell activation occurs.

It has been estimated that subjects infected with HIV, but not yet diagnosed as having AIDS, lose approximately two billion CD4 cells each day. While some of these cells will be replaced by the subject's own immune system, cell numbers eventually decline. Additionally, free gp120 may interact with uninfected CD4 cells of HIV-infected subjects, thereby reducing the effectiveness of surviving CD4 cells. It has therefore been proposed to produce large populations of CD4 cells for transfusion into HIV-infected subjects to replace cells destroyed or inactivated due to infection.

CD8 cell levels in the blood of HIV infected subjects are typically near normal. However, cytotoxic T lymphocyte ("CTL") activity is generally impaired in AIDS patients. Cell mediated immune responses are the principle immunological defense to HIV infection and a vigorous CTL response early in infection has been associated with a lower rate of disease progression. CTL's are the major effector cells in this antiviral response. However, the decline in CTL activity observed following HIV infection suggests that anti-HIV activity by

CTL's is impaired. Thus, it desirable to have a means to induce the formation and/or proliferation of CTL's, and, even more preferably, the formation of HIV-specific CTL's. It would also be highly desirable to have a means of expanding CD4 cells, CD8 cells and $CD4^+$ $CD8^+$ T-cells ("DP cells") from HIV infected subjects, particularly if this could be accomplished while keeping viral levels in the cultured cells low. DP cells represent an early stage in T cell development and can mature to form CD4 cells or CD8 cells having varying antigenic specificities.

In order to maximize the effectiveness of treatment with expanded T cells, it is desirable that the infused cells be MHC compatible with the subject's tissue. Ideally, the best MHC match will be obtained by using the subject's own cells for infusion. However, it would be inadvisable to remove large numbers of cells from the blood of an HIV-infected patient for culture or expansion, as this may further compromise the subject's ability to mount effective immune responses against foreign antigens.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method to expand a population of T cells from HIV-infected subjects which will allow the production of enough cells to be effective in bolstering the subject's immune response from an initial sample which is small enough so that its removal does not pose a significant health risk to the subject.

It is a further method of the invention to provide a method of expanding a cell population containing HIV-infected cells which does not lead to the production of high levels of HIV in the expanded population.

It is a further object of the invention to provide a method to interfere with the expression of HIV DNA in the expanded cell population.

It is a further object of the invention to provide a use of CM to inhibit the expression of HIV DNA in a cell infected with an HIV provirus.

It is a further object of the invention to provide a method of screening cell populations derived from HIV infected subjects for susceptibility to one or more anti-HIV treatments.

It is a further object of the invention to provide a use for CM in screening cells from HIV infected subjects for susceptibility to one or more anti-HIV treatments.

It is a further object of the invention to provide a use for CM to generate cell banks.

It is a further object of the invention to provide a composition of matter comprising an expanded population of CD8 cells derived from an HIV infected patient.

It is a further object of the invention to provide a composition of matter comprising an expanded population of DP cells derived from an HIV infected patient.

It is a further object of the invention to provide a use of CM to obtain a late culture cell population from a T cell sample obtained from an HIV infected subject.

It is a further object of the invention to provide a composition of matter comprising a substantially pure late culture population derived from a T cell population obtained from an HIV infected subject.

BRIEF DESCRIPTION OF THE DRAWINGS

In the accompanying drawings:

FIGURE 1 is a graphical depiction of the results of Example 3 examining the expansion of cell populations enriched for CD4 cells and CD8 cells obtained from three HIV infected subjects and cultured in CM/P or PHA/IL-2.

FIGURE 2 is a graphical depiction of the results of Example 3 examining the expansion in CM/P of a cell population obtained from HIV infected subjects and enriched in CD4 cells.

FIGURE 3 is a is a graphical depiction of the results of Example 3 examining the expansion in CM/P of a cell population obtained from HIV infected subjects and enriched in CD8 cells.

FIGURE 4 is a graphical depiction of the results of Example 3 examining the expansion in CM/P and PHA/IL-2/P of CD4 cells and CD8 cells obtained from HIV infected subjects.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As embodied and broadly described herein, the present invention is directed to methods for the expansion of CD4, CD8, and DP cell populations with reduced levels of HIV from cells derived from HIV infected subjects. In this application, the phrase "reduced levels of HIV" means a lower viral load or lower supernatant $p24^{gag}$ ("p24") level per viable T cell than what is observed in a sample from the same source, cultured in a similar manner, in RPMI-1640 medium supplemented with 10% heat inactivated pooled normal human serum (from non-HIV infected donors) and soluble anti-CD3 antibodies (1 μg/ml) (as described in Moran, *AIDS RESEARCH and HUMAN RETROVIRUSES* 9:455 (1993). The quantity of soluble p24 in cell culture supernatants is a common measure of HIV levels. Soluble p24 levels may be determined by standard methods. A preferred method is ELISA. The ELISA detection limit for p24 varies somewhat depending on reagents and conditions but is typically approximately 0.005 ng/ml. The viral load is another common measure of the levels of the HIV *gag* sequence from cell culture supernatants. The levels of HIV *gag* in supernatants may be determined by standard methods. A preferred method is reverse-transcriptase PCR ("RT-PCR") amplification which typically had a detection limit of about 400-500 HIV *gag* copies/ml.

The present invention is also directed to a method for screening populations comprising HIV infected cells obtained from a particular patient to assess the effectiveness of various antiviral treatments, including the *ex vivo* culture of T-cells in CM. Additionally, the present invention is directed to methods for the inhibition HIV gene expression.

Current methods of cell expansion cannot generate large expanded populations of both CD4 and CD8 T cells from a single initial sample obtained from an HIV positive subject. Surprisingly, it has been discovered that culture of a small initial mononuclear cell population derived from HIV infected subjects in the presence of a conditioned medium (CM) allows the large expansion of populations of CD4 cells, CD8 cells, and DP cells. This allows the production of cell banks containing T cells of different types suitable for use in immune reconstitution. Even more surprisingly, it has been discovered that following expansion in CM, the viral load in the resultant expanded population is low. In relative terms, the HIV viral load or supernatant p24 levels observed in cell populations from appropriate subjects expanded by the method of the invention are lower than the levels observed in cell populations from the same source expanded in RPMI-1640 medium containing 10% heat inactivated pooled normal human serum (from non-HIV infected donors) and 1 μg/ml soluble anti-CD3 antibody. In absolute terms, supernatant p24 levels in suitable subjects are below 1 ng/ml and in very suitable subjects they are equal to or less than 0.1 ng/ml. Extremely suitable subjects, the culture of whose cells by the method of the invention yields supernatant p24 levels below 0.005 ng/ml, are often observed. It is possible to identify appropriate patients, suitable patients, very suitable patients, and extremely suitable patients using the screening method of the invention.

The present invention also teaches a method of screening T cell populations derived from individual HIV-infected patients to determine their responsiveness to different anti-viral therapies, including culture in CM, and treatment with other antiviral agents such as human plasma and drugs including reverse transcriptase inhibitors and protease inhibitors such as Zidovidine, Lamivudine, Indinavir, Zerit, Saquinavir, Nelfinavir, and Ritonavir, separately or in combination.

Conditioned Medium (CM) and Its Production

The conditioned medium CM used in the process of the present invention comprises a mixture of cell factors having a balance of stimulatory and inhibitory effects favouring the proliferation of the desired cell population. The CM composition is produced by treating a starting cell population with an inducing agent which includes at least one plant mitogen. Preferred plant mitogens include plant lectins such as concanavalin A (ConA) or phytohemagglutinin (PHA), and T-cell mitogens such as mezerein (Mzn) or tetradecanoyl phorbol acetate (TPA). Especially preferred is a combination of ConA and Mzn. Other mitogens of non-plant origin, including interferons of various kinds, may be used in addition. The starting cell population used to prepare the CM may comprise peripheral blood cells, umbilical cord blood cells, bone marrow cells, mixtures of two or more types of such cells, or fractions or mixed fractions of such types of cells. The starting cell population may be induced by adding the inducing agent(s) to an appropriate suspension thereof in aqueous, nutrient-containing medium. The CM inducing process may be affected by factors produced

by the cells during culture, and by culturing conditions such as the medium used, temperature, time of culture, pH, exogenous recombinant growth factors, nutrients, etc. The medium used may be serum free.

Blood cells used in the preparation of CM may be derived from healthy subjects or HIV infected donors and may be further enriched in mononuclear cells such as those obtained in the buffy coat fraction or by density gradient centrifugation. The T cells to be expanded ("initial population") and the starting cells used in the preparation of CM ("starting population") may be the same or different cell population. Furthermore, the cells may be derived from the same or different donors. CM may be prepared using starting cells from a patient obtained from one blood sample and the target lymphocyte population may be obtained from the same patient on the same or a second visit. Where the starting population is derived from an HIV infected subject, it is preferable to selectively remove HIV virions from the CM prior to use.

When used to expand autologous T cells, patient-derived CM has the added advantage of allowing the entire procedure to use biological components derived from only one person. The culture conditions and methods may be varied to produce a desired CM or the desired T cell proliferation. CM is typically prepared from approximately 50 ml of whole peripheral blood, over a culture period of 4 days. Target T cells are typically derived from between 10 and 50 ml of low density mononuclear cells and are expanded over a period of between 1 and 4 weeks.

Unlike conventional methods in which the significant expansion of only CD4 cells occurs, the present invention also allows the expansion of populations of CD8 and DP cells. CD8 cells may be expanded together with significant levels of CD4 cells in the same culture. Alternately, CD8 cells may be preferentially expanded in a separate culture. A preferred method for the preferential expansion of CD8 cells is the positive selection of CD8 cells from LDMNC's by conventional means prior to culture in CM. The addition of P further enhances the preferential production of CD8 cells, as does culture for 3 to 4 weeks.

T cells may be expanded from subjects having reduced T cell counts. However, as the method of the invention allows the expansion of an initial cell population, the higher the actual number of cells capable of forming target cells in the initial population, the higher the absolute number of target cells which can be produced by the method of the invention over a fixed time period. Thus, while the large rate of cell expansion provided by the method of the invention allows the production of therapeutically useful numbers of CD4 cells from subjects with CD4 cell counts as low as 50 cells/μl, higher CD4 counts are preferable. In a preferred embodiment of the invention the subject providing the initial population has >100 CD4 cells /μl. In a more preferred embodiment of the invention, the subject has >200 CD4 cells/μl, even more preferable >400 CD4 cells/μl. In a yet more preferred embodiment, the subject has >800 cells/μl. Similarly, it is preferable that the subject providing the initial population have CD8 cell counts >200 cells/μl, more preferably >400 cells/μl, and even more preferably >800 cells/μl.

In order to examine the mechanisms contributing to the low viral loads observed in cell populations expanded in CM, the impact of culture in CM on the expression of a reporter gene driven by the HIV LTR promoter was examined. These experiments reveal that culture in CM represents a potent means of inhibiting the expression of HIV DNA. In this application the term "HIV DNA" refers to genetic material encoding components of HIV which are incorporated into the genome of a host cell, and "expression" refers to transcription or translation by components of the host cell's transcription or translation machinery.

The present invention discloses that the culture of samples containing HIV infected cells in CM not only allows the production of CD4 cell populations expanded to a greater extent than is possible using previously known techniques, but also allows the significant expansion of cell types different from those which could be expanded using previously known techniques, namely CD8 cells and DP cells. Surprisingly, it is possible to expand CD4, CD8 and DP cells together in a single culture using the method of the invention.

T cell populations expanded by the methods of the invention may be further treated to deplete free gp120 from the culture medium, thereby enhancing the survival and activity of the expanded T-cells. Methods employing gp120 binding agents for the selective binding of gp120 are known in the art, and are within the capacity of a skilled technician. For example, anti-gp120 antibodies may be immobilized on a solid support and placed in the culture medium. Free gp120 will tend to bind to the immobilized antibodies and may be removed from the solution by removal of the solid support. In some circumstances the depletion of free gp120 from the culture medium will allow the enhanced survival and reactivity of

expanded cell populations. Furthermore, in some instances it will be desirable to deplete the expanded cell populations of HIV infected cells having gp120 on their cell surface using standard methods for the selective binding of gp120. Solid supports may be formed from any biologically acceptable material; however, preferred embodiments include immobilization of gp120 binding agents on the surface of tissue culture plates, and/or on magnetic beads.

Expanded populations containing T cells can be administered to HIV infected subjects to boost their capacity to respond to immunological challenge. Such expanded cell populations may also be modified prior to introduction into the subject to provide a population with desired immunological properties. For example, cells may be exposed to HIV antigens in the presence of MHC compatible antigen presenting cells to induce the activation of cells recognizing the antigen of interest. Potential antigens of interest include, but are not limited to, HIV proteins (such as gp160, gp120, and p24) or peptides or fragments thereof, such as the V3 loop of gp120. In light of the genetic diversity between HIV isolates, it may be desirable to expose cells to a range of HIV antigens from different HIV isolates. Alternatively, it will frequently be possible to induce the activation of HIV-specific cells using an entirely autologous system. For example, a blood sample may be obtained from an HIV infected subject which contains the antigen of interest, suitable antigen-presenting cells, and the cell population to be stimulated. The use of entirely autologous materials reduces the risks and complications associated with using blood components derived from other donors, and it may increase the therapeutic effectiveness of the activated cells by ensuring that the HIV epitopes they recognize are present in the HIV isolate infecting the subject to be treated.

The present invention also provides from another aspect, a means of culturing T cells from HIV infected subjects and assaying the effectiveness of potential antiviral treatments such as culture in CM and/or the use of drug combinations to control viral load. Due to the reduced number of CD4 cells in most subjects infected with HIV, it is not desirable to remove the large numbers of cells needed to generate sufficiently large quantities of cells desirable for drug assay using conventional methods. However, a method allowing the selective culture and expansion of T cells from HIV infected subjects allows such screening. Additionally, the screening method of the present invention allows the monitoring of the response of an HIV infected subject to ongoing drug treatment. For example, it is possible to routinely remove and culture a small sample of LDMNC's from a subject receiving a particular treatment to determine the ongoing effectiveness of that treatment on its own or compared to other alternative treatments. This allows the rapid detection of acquired resistance, thereby facilitating effective disease management on a patient-by-patient basis. Furthermore, the effect of a particular treatment on the subject's immune response may be assessed by examining the variety, specificity, and anti-HIV activity of cells in the sample. Moreover, unlike previous methods, the present method allows the rapid and efficient expansion of numerous cells types, including CD4, CD8, and DP cells. Thus, unlike previous methods, the present method allows the generation of a population for screening which closely resembles the actual cell population existing in the subject in need of treatment. This is extremely valuable, as it is believed that the levels and activity of both CD4 cells CD8 cells are important in disease prognosis and HIV progression.

The screening method of the present invention may preferably be conducted by the removal of a small blood sample from a patient followed by enrichment of LDMNC's by standard methods as described in Example 2. The LDMNC's are cultured in substantially the same manner as that described in Example 2, except that where only short-term culture is anticipated and large numbers of cells are not required, the LDMNC's may be plated out into 48 or 96-well tissue culture plates to facilitate the study of a larger number of drug combinations. Alternatively, the LDMNC's may be cultured for one or two passages (approximately 4 to 14 days) without the addition of antiviral materials other than CM, following which time the cells may be harvested, diluted to an appropriate level as described in Example 2, and plated out in 24 well plates.

Appropriate levels of various treatments of interest may be commenced immediately after plating out the cells, or the cells may be allowed to remain untreated for 1 to 2 days following plating prior to the commencement of treatment (other than 5 %CM which is preferably present in the medium at the time the cells are plated out). The cell count and HIV levels may be determined by standard methods at various times after plating out. The relative abundance and activity of different T-cell types may also be determined at various times by methods known in the art. In cases where it is desirable to screen the impact of particular treatments on one of CD4 or CD8 cells, the desired cell type may be isolated from the LDMNC's prior to plating out using conventional methods.

Additionally, cells may be subjected to gene therapy *ex vivo* to allow the expression of gene products of interest. Such modified cells may be introduced into a subject as a means of

therapy or diagnosis. Methods of gene therapy are known in the art. Briefly, expressible genetic material encoding a product of interest is introduced into cells. The treated cells are then screened for the presence of the stably integrated and expressible gene of interest. Cells having the desired characteristics are introduced into an appropriate region of the patient by appropriate means. For example, in some circumstances it may be appropriate to administer cells by intravenous infusion.

Products of interest may include proteins, peptides, and fragments thereof which, when expressed intracellularly, are capable of inhibiting HIV replication. Examples of such compounds are chemokines such as RANTES, MIP-1α, MIP-1β, proteases capable of cleaving viral proteins and rendering them ineffective, protease inhibitors which inhibit the normal post-translational processing of HIV polyproteins, and peptide antagonists of Tat. In some circumstances, products of interest will include antisense RNA complementary to a region of viral RNA and capable of interfering with the formation of virus particles, RNA complementary to an HIV gene expression regulatory element region and capable of interfering with the interaction between this region and its corresponding transcription mediating protein sequence, and ribozymes capable of inhibiting viral replication.

It will also be desirable in some instances to introduce exogenous proteins into expanded cells prior to infusion. Methods for doing this are known in the art. For example, one commercial system, known as "VP22" allows the introduction of fusion proteins into cells. The fusion proteins localize to the nucleus of the cell, where, depending on the protein introduced, they

can function to modulate processes occurring in the nucleus, such as aspects of gene expression.

Expanded cell populations may be used immediately, or may be cryopreserved for later use. The method of the present invention is broadly applicable to the expansion of CD4, CD8, and DP cells. An early step in this method comprises the selection of a desired source of target cells. In the practice of the method described herein, one or more types of T cell present in an original cell population can be preferentially expanded to enrich the fraction of the selected lymphocyte in the expanded cell population. Furthermore, desired T cell types may be expanded from an initial cell sample comprising the precursors of the desired cell type. Thus, T cell types present at very low levels in a cell sample can be selectively proliferated to increase their representation in the expanded population. During this expansion of the desired T cells, non-T cells can be allowed to die off, to remain unexpanded, or to fall in number and/or proportion in the expanded culture. One important clinical advantage of the method of this invention is that cell populations containing a fraction of the selected T cells can be produced simply and, in many cases, without the need for separation or purification steps. The method allows for the selective, sequential production of CD4 and CD8 T cells from a single unenriched blood or tissue sample. The present invention further provides a means of expanding a population of DP cells to generate subsets of CD4 cells and CD8 cells. Methods for the generation of CD4 cells and CD8 cells from DP cells are known in the art and it is within the capacity of a competent technician to generate such cells from DP cells as part of the process of the present invention.

An initial population for expansion in CM can be selected from any primary human lymphocyte source. Examples of potential sources include peripheral blood, umbilical cord blood, bone marrow, lymph nodes, thymus, spleen, Peyer's patches or other lymphocyte-containing tissue. Target cells may also be selectively expanded from sources enriched in leukocytes such as material obtained by leukapheresis. Lymphocytes may be derived from healthy subjects or from patients such as those infected with HIV.

Expanded cell populations obtained at each passage may be used directly in immunotherapy of the patient, may be modified further by subculture or genetic manipulation, or may be cryopreserved using standard techniques for use at a later time. Cell banks may be established from patients early after diagnosis of HIV infection as a means of providing autologous T cells for use in the later stages of disease where the immunodeficiency is more pronounced and certain cell types have been irrevocably lost from the patient's blood cell population.

T cells cultured in CM may be further modified by the addition of exogenous recombinant growth factors which promote the proliferation of a T cell population having a desired phenotype or function. Other agents may be added, including growth factors, selective chemical or biological inhibitors of cell growth, and other antiviral agents that interfere with or suppress HIV replication such as chemokines. Antiviral drugs may also be included in the culture medium as a further step to inhibit viral replication. In light of the present invention it is within the skill of a competent technician to determine what additional antiviral drugs are appropriate. Examples of potentially useful drugs include reverse transcriptase inhibitors and

protease inhibitors such as Zidovidine, Lamivudine, Indinavir, Zerit, Saquinavir, Nelfinavir, and Ritonavir. In some instances Fluconazole will also be useful. Furthermore, the target cell population may be cultured in CM in the presence HIV antigens of interest and appropriate antigen presenting cells and/or target cells to promote the expansion of HIV-specific CD4 and CD8 cells. Such HIV-specific cells may subsequently be infused into an MHC compatible subject who is infected with a strain of HIV expressing epitope recognized by the HIV-specific cells in order to delay HIV progression in that subject.

The Expanded Cell Population

Cultures of T cells expanded in CM typically experience a 4 - 5 log expansion in 3 - 4 weeks of culture. CD4 cells, CD8 cells, and DP cells may be obtained, depending on the culture conditions and culture time employed. At each passage the relative abundance of different T-cell types may be assessed using standard techniques. For example, in typical cultures of low density mononuclear cells ("LDMNC's"), CD4 cells predominate during the first two weeks of culture and CD8 cells predominate thereafter. Thus, cultures expanded in CM for two weeks may be used to provide a population that is enriched in CD4 cells. Conversely, cultures expanded for 3 - 4 weeks may be used to provide a population enriched in CD 8 cells. All cultures may be serum and plasma -free medium, or may be supplemented with autologous or pooled human serum or plasma. Plasma-containing cultures typically convert more quickly and completely from a CD4 dominated population to a CD8 dominated population. Accordingly, plasma may be used to provide both a greater overall T cell expansion, and to provide purer populations of the desired cells.

T cell populations which have been enriched for CD4 cells, CD8 cells, or DP cells may also be expanded in CM. Relatively pure populations of CD4 cells and CD8 cells can be maintained in culture and used to treat subjects as required. Such enriched T cells expand 3 - 9 logs in 6 -7 weeks, permitting the production of large numbers of therapeutically effective CD4 and CD8 T cells from a small blood sample. Such large cell population expansion also permits the creation of cell banks suitable for use in delayed and repetitive infusions. Populations enriched in CD4 cells and CD8 cells may be recombined prior to infusion where desirable to obtain enhanced therapeutic efficacy.

Uses of the Expanded T Cell Populations

Expanded T cell populations may be used to treat HIV-infected subjects. A subject's own cells may be used to reconstitute his or her immune system throughout disease progression by autolymphocyte therapy. Repetitive or cyclical infusions of autologous T cells derived from cells extracted prior to the onset of significant immunodeficiency can serve as an effective means of fortifying immunity during the later stages of HIV infection when certain T cell populations in the subject's body have been irrevocably lost. The unique ability of CM to expand human umbilical cord blood T cells also permits such cells to serve as a source of lymphocytes for the treatment of HIV infected subjects, provided that the cells are appropriately matched for histocompatability. For example, HIV infected babies may be treated with T-cells derived from their own umbilical cord blood. Naive T cell populations derived from cord blood may be established for the treatment of HIV infected subjects. Thus, culture in CM permits the use of universal donor lymphocyte banks for immune reconstitution in HIV infection and AIDS.

T cells cultured according to the method of the present invention may be used in a method of screening potential antiviral treatments. Differences between HIV isolates from different subjects can result in inter-patient variability in the effectiveness of many anti-viral strategies, including culture in CM as a means of inhibiting viral replication (excellent expansion of T-cell populations in CM is consistently observed).

It is frequently difficult for physicians to predict in advance what antiviral strategies will be effective in combating the HIV isolate infecting a particular patient. The present invention provides a means for the *in vitro* culture of cells from a HIV infected patient and an assessment of the effectiveness of the *ex vivo* culture in CM, as well as both *in vivo* and *ex vivo* treatment with various drug combinations on virus levels. In particular, T cells may be obtained from an HIV infected patient and cultured in multiwell plates in a culture medium comprising CM. The levels of supernatant $p24^{gag}$ ("p24") and viral RNA levels may be assessed by standard means. Those patients whose CM-cultured cells have peak p24 supernatant levels of less than 1 ng/ml are suitable patients for large-scale T-cell expansion in CM with or without the use of additional anti-viral agents. Additionally, different drugs or drug combinations of interest may be added to different wells and the impact on virus levels in the sample may be assessed by standard means. Those drug combinations providing adequate viral suppression are therapeutically useful combinations. In light of the invention it is within the capacity of a competent technician to determine what constitutes adequate viral suppression in relation to a particular subject. In order to test the effectiveness of drugs of interest in limiting viral expansion, additional factors such as anti-CD3 antibodies may be added to the culture to stimulate viral production. Unlike culture methods for HIV infected

cell samples known in the art, CM allows the culture of T cells for periods of over two months, thereby providing an effective system in which to assay long term drug effectiveness.

T cells produced by the expansion of cells obtained from HIV infected subjects may also be used to treat certain opportunistic infections and tumours directly. Diseases of interest include pneumocycstus carnii, CMV, Kaposi's sarcoma and nonHodgkin's lymphoma. Subject-derived antigen-specific cytolytic T cells can be generated by co-culturing a population of subject-derived T-cells with MHC compatible antigen presenting cells and/or target cells in the presence of the antigen of interest. Antigen presenting cells, target cells, antigen-presenting cells, effector cells, and antigen may be endogenous and patient-specific.

Bone marrow transplants may be used to treat HIV infection itself and/or malignancies resulting from HIV infection. Infusions of autologous lymphocytes, expanded in CM, may be used to support bone marrow transplantation and to reduce the toxicity and risk of infection associated with the procedure.

The method of the invention allows the inhibition of gene expression driven by the HIV LTR promoter region in a cell population by the culture of cells in a medium comprising the CM according to the method of the invention. While it is not intended that the invention should be limited to any theory, it is suspected that culture in CM inhibits HIV LTR driven gene expression by altering one or more interactions between transcription mediating proteins and HIV gene expression regulatory elements. Transcription-mediating proteins of interest include host cell encoded proteins such as AP-1, NFkappaB, NF-AT, IRF, LEF-1 and Sp1,

and the HIV encoded protein Tat. HIV gene expression regulatory elements of interest include binding sites for AP-1, NFkappaB, NF-AT, IRF, LEF-1 and Sp1, as well as the transacting responsive element ("TAR") which interacts with Tat.

The range of viral levels observed from experiments involving the culture of HIV infected cells obtained from different patients likely reflects, in part, different mutations in the genes encoding transcription mediating proteins and/or HIV gene expression regulatory elements, which compensate for the inhibitory effect of culture in CM according to the method of the invention. Thus, in a preferred embodiment, the HIV infected cells are obtained from a subject with susceptible transcription mediating protein sequences and susceptible HIV regulatory element sequences. In a more preferred embodiment, the HIV infected cells are obtained from a subject having wild-type transcription-mediating protein sequences and wild-type HIV regulatory sequences.

The relationship between Tat and TAR sequence and HIV gene expression has been extensively examined. For example, Garcia *et al.* (*Genes Dev* 5:2128 (1991)) disclosed that stem structure of TAR RNA stem loop, the primary sequence of the loop, and the bulge element are major determinants for Tat activation. As a further example, Kamine *et al.* (*Virology* 182:570 (1991)) disclosed regions of the Tat protein sequence important for binding to TAR and found that the basic region of Tat combined with 8 random amino acids allows specific binding of this peptide to TAR. The requirements for transactivation of HIV transcription by Tat were examined by Kuppuswamy *et al.* (*Nucleic Acids Research* 17:3551-61, (1989)). Kuppuswamy *et al.* identified four domains in the N-terminal region of Tat

important to transactivation. These and other articles in the prior art allow the assessment of the regions of Tat and TAR necessary for productive interaction. Methods of sequencing RNA and proteins are known in the art and it is within the skill of a competent technician to determine the TAR and Tat sequences in an HIV isolate. In addition to referring to the amino acid and RNA sequences of Tat and TAR, respectively, it is also possible to use methods of molecular modeling known in the art to predict if mutations in these sequences will significantly effect the interaction between Tat and TAR due either to conformational or steric changes or to changes in the phosphorylation pattern of the regions of interest. It is therefore well within the capacity of a technician skilled in the art to identify wild-type sequences and determine if a particular sequence is equivalent to a wild-type sequences.

Similarly, the sequences of transcription mediating proteins such as AP-1, NFkappaB, NF-AT, IRF, LEF-1 and Sp1, as well as their binding sites, are known in the art. The interactions necessary for productive binding of these transcription mediating proteins and their binding sites have been reported in the prior art and it is within the capacity of a competent technician to determine if a particular mutation will significantly affect the interaction between the transcription mediating protein and its corresponding HIV gene expression regulatory element using the techniques previously discussed with respect to the Tat and TAR sequences.

The phrase "equivalent to wild-type sequence" as used in this application refers to a sequence which differs from wild type sequences only in respect of one or more features which do not significantly affect the interaction between a transcription mediating protein and its HIV gene

expression regulatory element. The phrases "susceptible transcription mediating protein sequence" and "susceptible HIV regulatory element sequence" when used in this application, refer to wild type sequences and equivalent to wild-type sequences.

It is well known in the art that there are many strains of HIV and that different strains exhibit different characteristics and responses to treatment. It is to be expected, therefore, that some variability will be observed in the response of cell samples from different patients to culture in CM. However, unlike conventional approaches to HIV treatment which allow an assessment of effectiveness only after extensive treatment (during which time the HIV infection may progress substantially), culture in CM allows a means to determine the effectiveness of the proposed treatment quickly and efficiently.

Although it is known that culture in CM reduces HIV LTR driven gene expression, thereby reducing overall HIV replication, it is believed that several other aspects of CM contribute to its effectiveness. It is known that CM contains RANTES, MIP-1α, and MIP-1β which have been shown to inhibit the initial infection of cells by HIV. Additionally, unlike conventional methods for expanding HIV-infected cell populations, the present invention allows the expansion of significant numbers of CD8 cells and DP cells in the same culture as CD4 cells. CM supports the formation of cytotoxic T-lymphocytes from CD8 cells. Disease prognosis in HIV infection has been correlated to the level of active CD8 cells in the subject's blood. Finally, it is believed that there exist CD8-derived factors which play a role in inhibiting HIV replication (Kinter et al., *PNAS USA* 93(24):14076 (1996)). Thus, CM provides a unique

culture system which allows the production of CD8-derived anti-viral factors in a culture containing significant numbers of CD4 cells.

Thus, culture of LDMNC populations in CM is believed to reduce HIV expansion by several means, including: (a) reducing HIV gene expression through an indirect mechanism, (b) inhibiting the infection of cells by HIV indirectly, and (c) allowing the production of a CD8-derived antiviral factor.

The invention is exemplified and demonstrated in the following specific experimental examples.

EXAMPLE 1

CONDITIONED MEDIUM (CM)

XLCM™ - a Preferred Embodiment of CM

Human umbilical cord blood containing 20 units of heparin per ml was used as the starting material for the preparation of XLCM (a preferred embodiment of CM), as described in Skea, *Blood* 90:3680 (1997), Skea, *J. Hematotherapy* 8:129 (1999) and International Publication No. WO 98/33891. Briefly, blood cells were diluted and mezerein was added at a final concentration of 10 ng/ml and the mixture was incubated for 2 hours in a humidified incubator kept at 37°C and 5 % CO_2. Concanavalin A was then added at a final concentration of 20 µg/ml and the incubation was continued under the same conditions for four days. The supernatant XLCM was harvested by centrifugation at 500 x g for 30 minutes at 4°C, and

filtered using a 0.22μm syringe-mounted filter unit. For the stimulation of cells, XLCM was added to the culture medium at a concentration of 5 % by volume. In addition to whole cord blood, whole or buffy coat peripheral or leukapheresis blood from healthy donors or patients may be used to prepare XLCM, as required.

COMPOSITION OF XLCM

XLCM contains a unique balance of positive and negative regulators of T cell growth. At least 20 known growth regulating factors are present in significant quantities, including chemokines, interleukins, colony stimulating factors and cytokines. Factors of particular interest include: RANTES, MIP-1α, MIP-1β, IFN-γ, and IL-2. It is also noteworthy that XLCM contains only very low levels of IL-6 and IL-4. The relative amounts of several growth regulating factors in XLCM are shown in Table 1, grouped according to concentration as measured by enzyme-linked immunosorbent assay (ELISA). No combination of the recombinant forms of these regulatory factors has been found which reconstitutes the growth promoting activities of XLCM.

EXAMPLE 2 EXPANSION OF T CELLS FROM THE LOW DENSITY MONONUCLEAR CELLS OF HIV INFECTED DONORS

Low density mononuclear cells were prepared from the peripheral blood of HIV infected donors at various stages of disease by density fractionation. These patients had been on a range of drug therapies prior to the donation of these blood samples. Samples of 10 - 30 ml of whole blood were layered onto an equal volume of a Ficoll-Hypa discontinuous gradient

(density 1.077 g/ml) in 50 ml conical tissue culture tubes and centrifuged at 400 x g for 30 minutes at room temperature. The interphase material containing LDMNC was collected, and the cells were washed twice in serum-free medium (AIM-V (Sigma) containing 20 units/ml heparin and 50 μM 2-mercaptoethanol, hereinafter "HBCM-2") by centrifugation at 200 x g for 10 minutes at room temperature. The LDMNC were resuspended in HBCM-2, a sample was diluted 1:20 with 2 % acetic acid, and the cell count was determined by hemocytometer. The yield of LDMNC per ml of uninfected adult peripheral blood is typically between 1 x 10^6 and 10 x 10^6 (average 1.2 x 10^6). The yield of LDMNC per ml of blood from HIV infected subjects varies, but has been found to be between 0.2 x 10^6 and 3 x 10^6 (average 1.5 x 10^6). The initial CD4 cell counts from the HIV infected donors examined ranged from 70 to 740 CD4 cells per μl blood plasma before LDMNC isolation.

The LDMNC were diluted to a final density of 1 x 10^5 cells/ml in HBCM-2 containing 5 % XLCM prepared according to the method of Example 1. LDMNC were cultured in the presence or absence of 5 % human umbilical cord blood plasma ("P") in 24-well tissue culture plates (1.5 ml/well) and maintained at 37° C and 5 % CO_2 in a humidified incubator. No antiviral drugs were present in the *in vitro* cultures. Every 4 to 7 days the cell count and viability were determined by mixing a sample of the culture suspension with a equal volume of 0.4 % trypan blue and counting the unstained (viable) and blue (non-viable) cells by hemocytometer. At each time point, the cells were subcultured by diluting an appropriate volume of the culture to a density of 1 x 10^5 cells/ml in fresh HBCM-2 containing 5 % XLCM, with or without 5 % P, as appropriate. The fold of expansion of the starting cell number was determined at each passage, and the theoretical total yield calculated assuming

the entire culture had been expanded. The cell number at each passage and the maximum fold of expansion of LDMNC from 7 different HIV infected donors are shown in Table 2. The initial CD4 cell count per μl of blood plasma before LDMNC isolation and time in culture are also shown.

Initial CD4 cell counts ranged from 140 cells per μl to 740 cells per μl. XLCM stimulated 1 - 4 logs of total cell expansion over a culture period from 25 to 44 days. Although the cells expanded well in 5 % XLCM alone, the presence of cord plasma significantly improved the level of cell expansion. Indeed, cells from 5 of the 7 HIV infected donors achieved greater than a 5 log expansion in the presence of cord blood plasma. Control LDMNC from three patients stimulated with 5 μg/ml phytohemagglutinin (PHA) plus 10 U/ml recombinant human interleukin 2 (IL-2), but not XLCM, failed to expand to the first passage.

At selected time points, cultured cells were stained with fluorescently-labeled antibodies to the CD4 and CD8 T cell co-receptors, and were analyzed for the respective antigen expression by flow cytometry. The percent of CD4 cells, CD8 cells, and DP cells in the cell populations derived from 5 of the 7 donors of Table 2 between days 12-28 of culture are shown in Table 3. Surprisingly, in addition to CD4 cells, it was possible to expand therapeutically useful levels of CD8 and DP cells.

EXAMPLE 3 EXPANSION OF ENRICHED CD4 AND CD8 CELLS FROM HIV INFECTED DONORS

CD4 cells were selected from LDMNC prior to culture using anti-CD4 antibody-coated magnetic beads (MiniMacs, Miltenyi Biotec). Positively selected CD4 cells ("CD4-enriched") were eluted from the beads and seeded at 1 x 10^5 cells per ml in HBCM-2 in 24 well tissue culture plates, and cultured in 5 % XLCM with or without 5 % P, or 5 μg/ml PHA plus 10 U/ml IL-2 with or without 5 % P. The CD4-depleted ("CD8 enriched") fraction was similarly cultured. The cells were passaged every 4 to 7 days, depending on their density. Nine passages represents approximately 6 weeks. At each passage culture supernatants and cell pellets were collected and stored at -70° C for the measurement of virus as described in Example 4.

Overall, the expansion of the enriched CD4 cells in the presence of plasma resulted in a 5 to 32-fold greater expansion than was observed with XLCM alone. Enriched CD8 cells expanded only in XLCM plus plasma.

Figure 1 depicts the results of expansion of enriched CD4 cells and CD8 cells obtained from three HIV infected subjects and cultured in CM/P or PHA plus IL-2. These cultures represent donors having a range of CD4 cell counts and viral loads. One donor had a relatively high CD4 cell count (530 cells/μl) and undetectable viral load (<500 copies per ml). The second donor had both a very low CD4 cell count (<150 cells/μl) and a low viral load (<400 copies/ml), while the third donor had both a very low CD4 cell count (80 cells/μl) and a very high viral load (534,000 copies/ml). Cells from these patient plus cells from several additional HIV infected donors were similarly cultured and analyzed and the results are depicted in Figures 2, 3 and 4. In all cases, XLCM stimulated a strong proliferation of both

CD4 and CD8 enriched T cells. The level of T cell expansion in CM/P was significantly greater than that achieved in PHA/ IL-2 or PHA/IL-2/P. T cells from donors with low CD4 cell counts and higher viral loads were difficult to culture for even one passage in PHA/IL-2. For cells derived from HIV infected subjects with a CD4 cell count close to normal (>400 cells/μl), the maximum CD4 T cell expansion was 4 orders of magnitude greater than the total expansion of the same cells stimulated by PHA plus IL-2. T cells from three healthy donors gave similar results, with CD4 cell expansions in CM/P also being four orders of magnitude greater than in PHA plus IL-2.

A comparison of Figure 1 with Figures 2, 3, and 4 reveals that XLCM surprisingly allows the expansion of late culture cell populations which are lost in PHA/IL-2 cultures with or without 5 % plasma. In particular, while both XLCM cultures and PHA/IL-2 cultures show a marked reduction in expansion around passage number four (corresponding to approximately day 16), the XLCM cultures continue to expand at a significant rate beyond this point, whereas the PHA/IL-2 cultures tend to become static or die. This effect of XLCM is apparent in respect of both CD4 cell populations and CD8 cell populations, although CD8 cell populations show stronger late culture growth. Moreover, the kinetics of cell population expansion for both CD4 cells and CD8 cells indicates that this late culture growth is due primarily to a distinct cell population which rises in significance after passage 4 and dominates the culture by passage 7. This indicates the presence of a distinct "late culture population" in the XLCM cultured cells which is not present in the PHA/IL-2 cultures. Thus, unlike PHA/IL-2, XLCM allows the continued expansion of subpopulations within both CD4 and CD8-enriched cultures.

The late culture population observed may represent HIV-free cells or HIV-resistant cells. A cell population enriched for this late-culture population may be obtained by monitoring the rate of cell expansion and the absolute cell numbers in an expanding cell population and harvesting the expanded cell population at or after the first passage in which a recovery of cell expansion rate is observed following the sharp decline. For example, for the cell population depicted in figure 2, a cell population enriched for the late-culture population could be obtained by harvesting the cell population at or after passage 5. A cell population comprising a substantially pure population of the late culture population may be obtained by harvesting the expanded cell population several passages after the cell population becomes enriched for the late culture population after the point where the total number of cells in the culture is at least 2 and preferably at least three orders of magnitude higher than it was at the time the culture became enriched for the late culture population. For example, for the cell population depicted in Figure 2, a substantially pure late culture population could be obtained by harvesting the cell population after passage 7.

The median fold expansion for CD4-enriched and CD8-enriched cell populations from 21 HIV infected subjects, broken down by CD4 cell count, are depicted in Table 4. HIV infected subjects were classified into three disease stages based on their peripheral blood CD4 cell count: (1) CD4 count above 400/mm^3 - still relatively healthy, (2) CD4 count between 200 and 400 cells/mm^3 - progressing towards AIDS, and (3) CD4 count below 200/mm^3 - AIDS. CD4 cells and CD8 cells were isolated and expanded with CM/P. These data represent the results of 44 experiments using 27 blood samples obtained from a total of 21 different

patients. Very large cell expansions were achieved even when the starting CD4 cell counts were very low.

EXAMPLE 4 SUPPRESSION OF HIV REPLICATION BY CM

Table 5 summarizes the CD4 and CD8 cell counts, viral load and peak soluble $p24^{gag}$ observed in expanded populations of CD4-enriched cells obtained from 16 patients. Viral load was determined by reverse-transcriptase polymerase chain reaction (RT-PCR) amplification of the HIV-1 RNA *gag* sequence from donor plasma, and is expressed as RNA copies per ml. The RT-PCR limit of detection was 400-500 copies/ml and for the purposes of this study measurements below this value are considered undetectable. CD4 cells were isolated and expanded with CM/P. At each passage, culture supernatants were collected and soluble HIV $p24^{gag}$ was measured by ELISA (Oigamon-Tecknika). The ELISA limit of detection was 0.005 ng p24/ml. The peak $p24^{gag}$ level measured over the course of each culture is reported. In 12 cultures, the concentration of $p24^{gag}$ was less than 1 ng/ml indicating very low levels of viral replication. In 6 cultures $p24^{gag}$ was undetectable. In only 2 cases were very high levels of $p24^{gag}$ observed, and these did not correlate with low CD4 counts or high viral loads.

Thus, XLCM can stimulate vigorous CD4 cell, CD8 cell and DP cell proliferation from HIV infected donors without high levels of virus replication. XLCM therefore offers a significant addition to current drug therapies for the treatment of HIV-infected individuals over a wide range of donors and disease states by permitting the *in vitro* expansion and reinfusion of their

own CD4 cells, CD8 cells, and DP cells as a means of reconstituting or maintaining their immune system.

EXAMPLE 5 SUPPRESSION OF HIV LTR-DRIVEN GENE EXPRESSION BY CM

The effect of XLCM on LTR-driven gene expression was studied on a human CD4 T cell line. 3×10^7 Jurkat cells were transfected by the DEAE-dextran DNA transfection method with 10 μg of a DNA construct containing the chloramphenicol acetyltransferase (CAT) gene under the control of the HIV LTR promoter together with 5 μg of a plasmid carrying the HIV *tat* regulatory sequence. Immediately after transfection the cells were placed in 25 % CM/10 % FBS/RPMI. Twenty-four hours later, cells were stimulated with 25 ng/ml phorbol myristate acetate (PMA) and 2 μM ionomycin for 18 hours, lysed, and the level of CAT expression was determined by ELISA.

HIV LTR-transfected Jurkat cells cultured in XLCM produced 950 pg CAT compared to 2,530 pg CAT in the absence of CM, representing a 62 % reduction. These data demonstrate that XLCM substantially suppresses LTR gene expression in a T cell line and indicate that XLCM also suppresses viral replication in primary cultures of T cells by suppressing HIV LTR-driven gene expression.

While the invention will be described in conjunction with illustrated embodiments, it will be understood that it is not intended to limit the invention to such embodiments. On the contrary, it is intended to cover all alternatives, modifications and

equivalents as may be included within the spirit and scope of the invention as defined by the appended claims.

Table 1: Cytokines in CM

Cytokine	Concentration* in CM (ng/ml)	Range (ng/ml)
IL-8	234	181 - > 1000
TNF-	112	98 - 160
MIP-1	98	68 - 243
IL-2	44	12 - 159
TGF-1	21	6.9 - 44
RANTES	15	4 - 54
MIP-1	11	1 - 39
GM-CSF	11	0.7 - 24
TNF-RII	9.1	6.8 - 17
IL-1	6.4	0.2 - 18
M-CSF	5.4	2.3 - 9.7
IL-13	3.6	1.5 - 13
IFN-	3.6	0.6 - 14
IL-1	2.3	0.004 - 4.9
IL-16	2.1	0.5 - 6
TNF-RI	1.8	1.1 - 2.4
Fas	1.3	< 0.04 - 2.3
TNF-	0.37	< 0.001 - 3.4
IL-12	0.26	0.07 - 0.8
SCF	0.2	0.15 - 0.29
IL-10	0.02	0.007 - 0.2
IL-6	0.007	< 0.006 - 0.028
IL-4	0.0068	0.00012 - 0.08

* median cytokine concentration measured in n = 6 -18 independent lots of XLCM™ using commercial ELISA kits: IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, RANTES, TGF-1, TNF-RI, TNF-RII, Fas (Biosource International, Camarillo, CA), INF- (Genzyme Diagnostics, Cambridge, MA), IL-16 (Immuno Diagnostics Inc., Buffalo, NY), TNF-, GM-CSF, MIP-1 (Intergen Co., Purchase, NY) and MIP-1TNF-IL-1SCF, M-CSF (R & D Systems, Minneapolis, MN).

Table 2. Expansion of LDMNC from HIV⁺ Donors in XLCM™

Donor	CD4 Count /ul	Condition	Viable Cells (x 10^{-5})/ml Passage Number						Maximum Fold Expansion	Culture Time, days
			1	2	3	4	5	6		
91-00119	140	XLCM™/P	8.6	5.6	21.4	1.8	-	-	1,855	25
		XLCM™	1.0	3.0	1.8	-	2.2		12	32
96-00570	160	XLCM™/P	17.8	25.0	17.6	8.0	3.2	4.0	801,997	41
		XLCM™	2.2	19.0	8.8	3.6	2.4	-	3,178	34
96-00555	180	XLCM™/P	1.2	23.8	21.2	7.4	4.8	3.4	73,122	39
		XLCM™	1.0	13.2	6.8	5.6	2.6	2.2	2,875	39
92-00233	400	XLCM™/P	9.2	19.8	22.4	9.4	7.2	3.2	883,713	41
		XLCM™	8.4	18.0	14.0	6.2	4.2	-	55,121	34
92-00265	430	XLCM™/P	12.6	21.6	35.8	14.4	6.4	-	897,945	32
		XLCM™	-	9.8	16.6	6.2	5.6	-	5,648	32
94-00391	440	XLCM™/P	13.8	25.4	23.8	10.2	2.5	1.7	361,642	41
		XLCM™	9.4	18.8	23.8	4.4	3.1	1.1	63,106	44
93-00378	740	XLCM™/P	19.2	52.8	40.8	48.8	-	-	2,018,437	28
		XLCM™	8.6	13.6	21.6	30.4	-	-	76,801	28

Table 3. Percent $CD4^+$ and $CD8^+$ T Cells in LDMNC from HIV^+ Donors

Donor	Condition	Time, days	% $CD4^+$	% $CD8^+$	% $CD4^+/CD8^+$ Double Positives
91-00119	XLCM™/P	12	22	83	8
		25	8	87	3
	XLCM™	12	42	46	7
		-	-	-	-
96-00555	XLCM™/P	12	43	69	15
		25	23	83	9
	XLCM™	12	52	43	6
		25	60	25	2
92-00265	XLCM™/P	12	67	42	11
		25	33	73	10
	XLCM™	12	69	16	3
		25	67	14	3
96-00570	XLCM™/P	17	34	79	14
	XLCM™	17	59	50	15
93-00378	XLCM™/P	15	53	38	12
		28	12	93	15
	XLCM™	15	66	27	4
		28	69	21	5

Table 4: Expansion of T Cells from HIV+ Patients At Different Stages of Disease

	Stage of Disease		
	CD4 Count > 400/mm^3	CD4 Count 200-400/mm^3	CD4 Count < 200/mm^3
CD4+ T lymphocytes Median Fold of Expansion Range n	8×10^4 2×10^3 - 2×10^8 9	6×10^4 2×10^2 - 2×10^5 6	9×10^4 2×10^1 - 2×10^9 11
CD8+ T lymphocytes Median Fold of Expansion Range n	1×10^6 1×10^5 - 7×10^7 4	1×10^5 1×10^4 - 3×10^5 5	4×10^6 1×10^4 - 7×10^9 9

Table 5: Viral Replication During Culture of HIV+ Patient T Cells with XLCM/P

Patient	CD4 Count (cells/ul)	CD8 Count (cells/ul)	Viral Load (copies/ml plasma)	Peak $p24^{gag}$ (ng/ml)
558	700	nd	< 500	0.006
116	540	nd	< 500	< 0.005
433	530	850	< 500	< 0.005
391	530	660	< 500	< 0.005
546	500	nd	< 500	1400
478	480	nd	25730	0.18
431	470	560	452	0.07
292	440	nd	< 500	1.45
233	400	1090	29010	< 0.005
632	390	nd	< 500	11.5
435	190	1270	< 500	< 0.005
268	180	1100	< 500	0.17
570	160	670	< 500	< 0.005
432	150	580	< 500	0.006
491	80	700	333000	900
634	70	250	638000	0.75

WHAT WE CLAIM AS OUR INVENTION:-

1. A method of obtaining an expanded population of target T cells with reduced levels of HIV from an initial population of T cells obtained from an appropriate subject infected with HIV, the method comprising:

(1) preparing a CM containing a combination of factors effective for promoting the expansion of the chosen target T cells by incubation of a starting population of blood cells in a growth medium containing at least two plant-derived mitogens; and

(2) culturing an initial population of T cells containing the target T cells or precursors thereof in the presence of an effective amount of the CM from step (1), to expand the population of target T cells in the culture while maintaining reduced virus levels.

2. The method of claim 1 wherein the CM is XLCM..

3. The method of claim 2 wherein the supernatant p24 levels are less than 0.1 ng/ml.

4. The method of claim 1 wherein the starting population of blood cells comprises human umbilical cord blood cells.

5. The method of claim 1 wherein the starting population of blood cells comprises human peripheral blood cells.

6. The method of claim 1 wherein the CM is prepared from a starting population of blood cells obtained from a subject not infected with HIV.

7. The method of claim 1 or 2 wherein the CM is prepared from a starting population of blood cells obtained from the same source as the initial cell population.

8. The method of claim 1 or 2 wherein the expanded cell population comprises a T cell type selected from the group consisting of CD8 cells, CD4 cells, and DP cells.

9. The method of claim 1 wherein the expanded cell population is further monitored during culturing to determine the relative levels of different T-cell types at each passage.

10. The method of claim 1 wherein the initial cell population is obtained from a subject infected with HIV-1.

11. The method of claim 1 wherein the initial cell population is obtained from a subject infected with HIV-2.

12. The method of claim 1 wherein the initial cell population is obtained from a subject infected with HIV-0.

13. The method of claim 1 wherein the initial cell population is obtained from a subject infected with an isolate of HIV having wild-type HIV gene expression regulatory element sequences.

14. The method of claim 1 or 2 wherein the initial cell population is obtained from a subject infected with an isolate of HIV having wild-type or equivalent to wild-type HIV gene expression regulatory element sequences.

15. The method of claim 1 wherein the initial cell population is obtained from a subject infected with an isolate of HIV having wild-type transcription mediating protein sequences.

16. The method of claim 1 wherein the initial cell population is obtained from a subject infected with an isolate of HIV having equivalent to wild-type transcription mediating protein sequences.

17. The method of claim 13 wherein the isolate of HIV further has wild-type transcription mediating protein sequences.

18. The method of claim 14 wherein the isolate of HIV further has wild-type or equivalent to wild-type transcription mediating protein sequences.

19. The method of claim 1 or 18 wherein the subject had a pretreatment CD4 count of >50 cells/μl.

20. The method of claim 1 or 18 wherein the subject had a pretreatment CD4 count of >100 cells/μl.

21. The method of claim 1 or 18 wherein the subject had a pretreatment CD4 count of >200 cells/μl.

22. The method of claim 1 or 18 wherein the subject had a pretreatment CD4 count of >400 cells/μl.

23. The method of claim 1 or 18 wherein the subject had a pretreatment CD4 count of >800 cells/μl.

24. The method of claim 1 or 18 wherein the subject had a pretreatment CD8 count of > 200 cells/μl.

25. The method of claim 1 or 18 wherein the subject had a pretreatment CD8 count of >400 cell/μl.

26. The method of claim 1 or 18 wherein the subject had a pretreatment CD8 count of >800 cell/μl.

27. The method of claim 1, wherein the culture medium further includes human plasma.

28. The method of claim 1 wherein the culture medium further includes human serum.

29. The method of claim 1, 2, or 4 wherein the medium further includes human cord blood plasma.

30. The method of claim 1 or 27 wherein the culture medium further includes at least one antiviral compound.

31. The method of claim 1 or 27 wherein the culture medium is further depleted of free gp120.

32. The method of claim 1 or 27 wherein the culture is further depleted of cells having gp120 on their surface.

33. The method of claim 1 wherein the target cells are additionally subjected to gene therapy.

34. The method of claim 1 wherein the target cell population is further exposed to one or more inhibitors of HIV replication.

35. The method of claim 34 wherein the inhibitor of HIV replication is an anti-viral drug.

36. The method of claim 1 wherein the target cell population is further exposed to a drug selected from the group comprising: Zidovidine, Lamivudine, Indinavir, Zerit, Saquinavir, Nelfinavir, and Ritonavir.

37. The method of claim 1 wherein the target cells are additionally subjected to exogenous protein introduction.

38. The use of CM in screening cells from HIV infected subjects for susceptibility to one or more treatments.

39. The use of claim 38 wherein the treatment is anti-viral drug treatment.

40. The use of claim 38 wherein the treatment is expansion in CM with reduced levels of HIV.

41. The use of claim 38 wherein the treatment is gene therapy.

42 The use of CM to generate cell banks.

43 The use of claim 42 wherein the cell banks are generated from umbilical cord blood.

44 The use of claim 42 wherein the cell banks are generated from peripheral blood.

45. The use of claim 42 wherein the cell banks comprise CD4 cells.

46. The use of claim 42 wherein the cell banks comprise CD8 cells.

47. The use of claim 42 wherein the cell banks comprise DP cells.

48. A composition of matter comprising an expanded population of CD8 cells derived from an HIV infected patient.

49. A composition of matter comprising an expanded population of DP cells derived from an HIV infected patient.

50. The composition of claim 48 or 49 wherein the expanded cell population contains reduced levels of virus.

51. The composition of claim 48 or 49 wherein the cell population has a supernatant p24 level of less than 1 ng/ml.

52. The composition of claim 50 or 51 wherein the expanded population of cells was obtained from a subject infected with an isolate of HIV having wild-type HIV gene expression regulatory element sequences and wild-type transcription mediating protein sequences.

53. The method of claim 1 or 2 wherein the expanded target cells are further exposed to an antigen of interest in the presence of MHC compatible antigen presenting cells.

54. The use of a CM to inhibit the expression of HIV DNA in a cell infected with an HIV provirus.

55. The use of a CM to obtain a late culture cell population from a T cell sample obtained from an HIV infected subject.

56. The use of claim 55 wherein the T cell sample is enriched in CD4 cells.

57. The use of claim 55 wherein the T cell sample is enriched in CD8 cells.

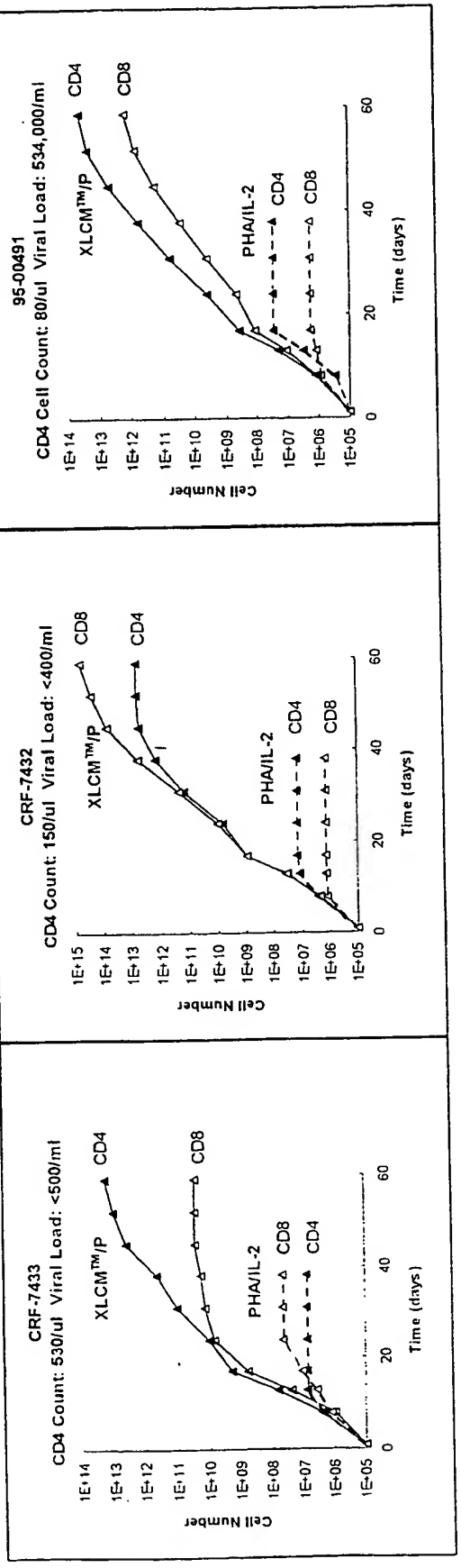
58. A composition of matter comprising a substantially pure late culture population derived from a T-cell sample obtained from an HIV-infected subject.

59. A method of obtaining an expanded population of target T cells with supernatant p24 levels of less than 1 ng/ml from an initial population of T cells obtained from a suitable subject infected with HIV comprising:

(1) preparing a CM containing a combination of factors effective for promoting the expansion of the chosen target T cells by incubation of a starting population of blood cells in a growth medium containing at least two plant-derived mitogens; and

(2) culturing an initial population of T cells containing the target T cells or precursors thereof in the presence of an effective amount of the CM from step (1), to expand the population of target T cells in the culture while maintaining reduced virus levels.

Figure 1. Expansion of Enriched CD4⁺ and CD8⁺ T Cells from HIV⁺ Donors by XLCM™



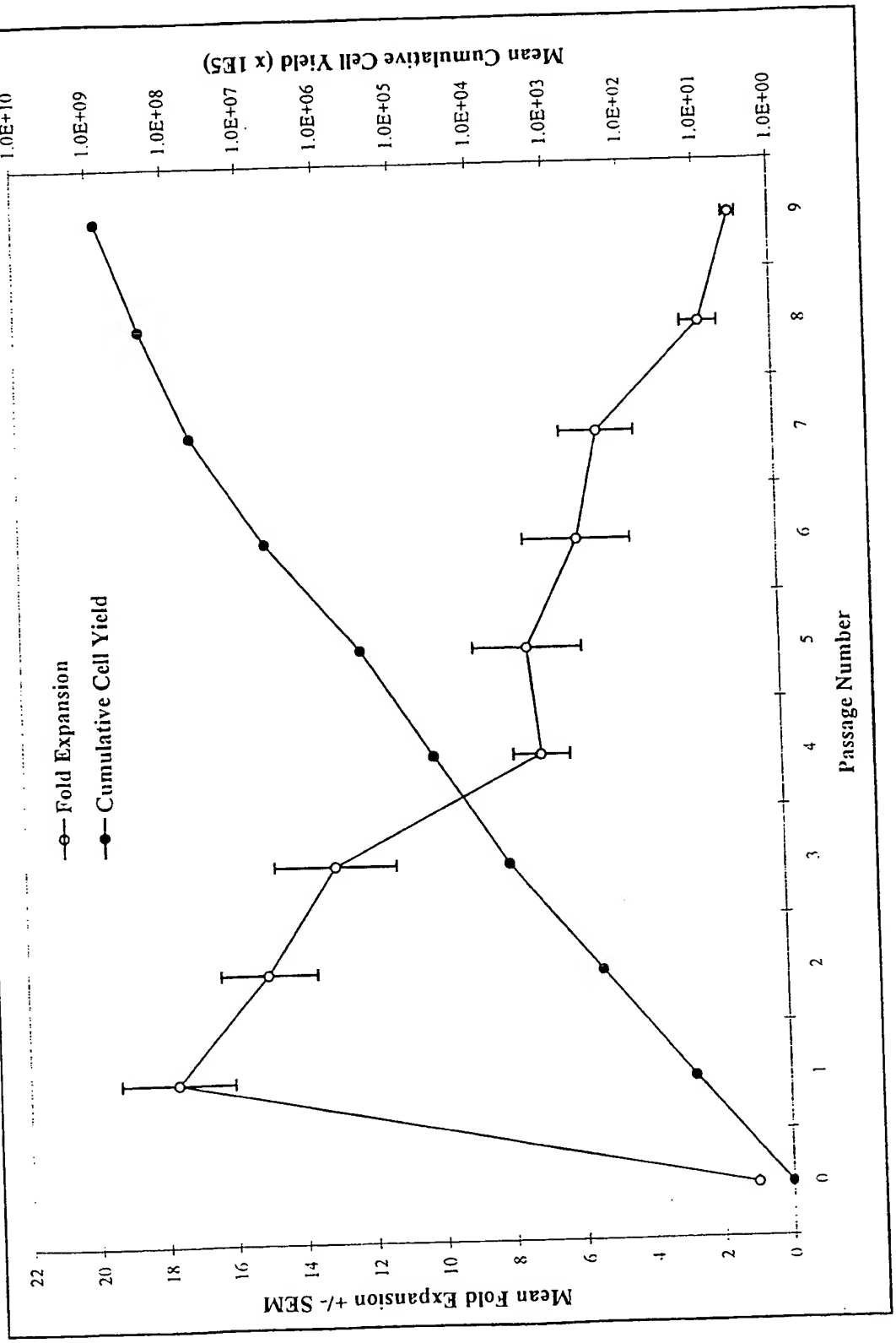


Figure 2: Expansion by XLCM/P of CD4+ T Cells from HIV+ Patients

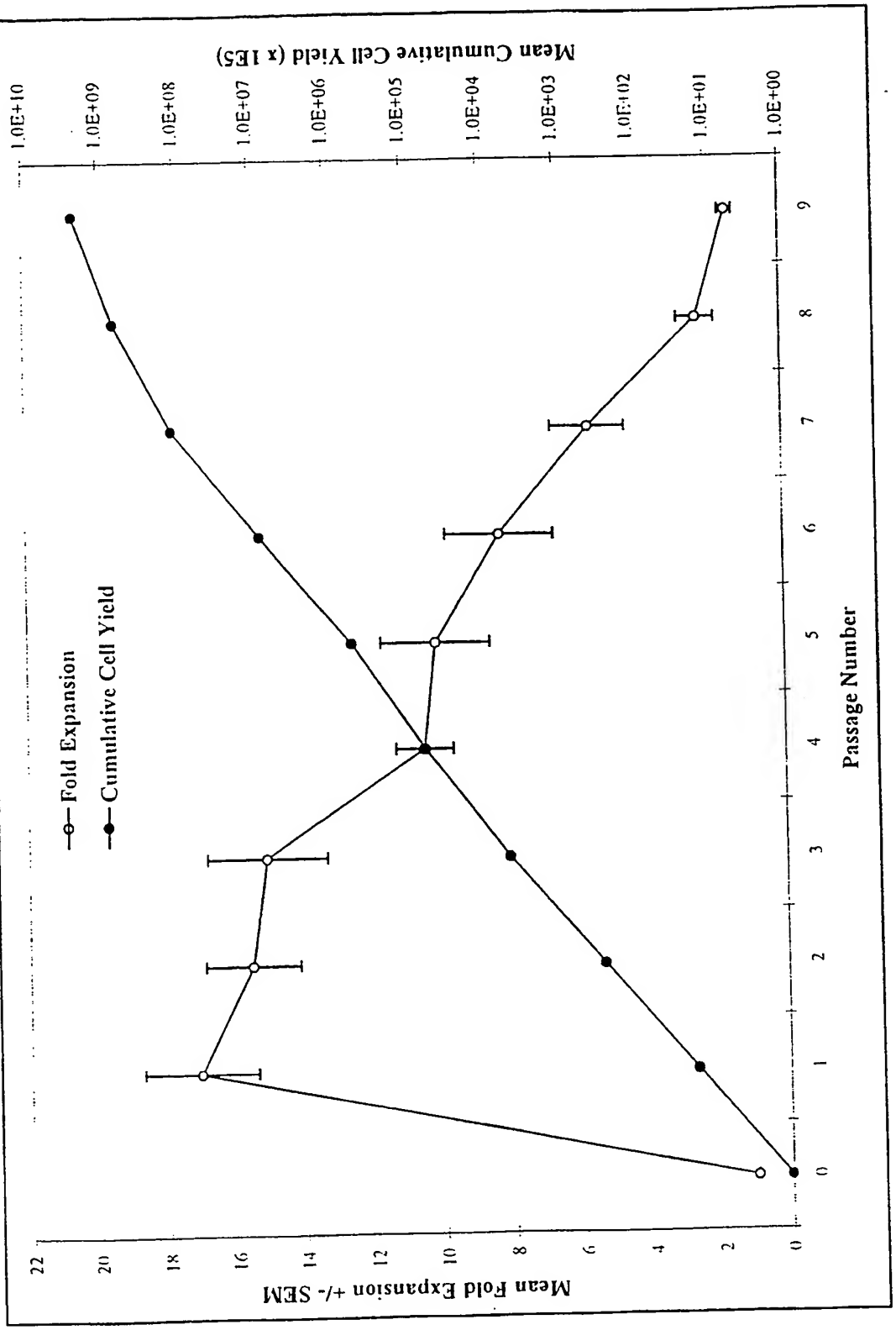


Figure 3: Expansion by XLCM/P of CD8+ T Cells from HIV+ Patients

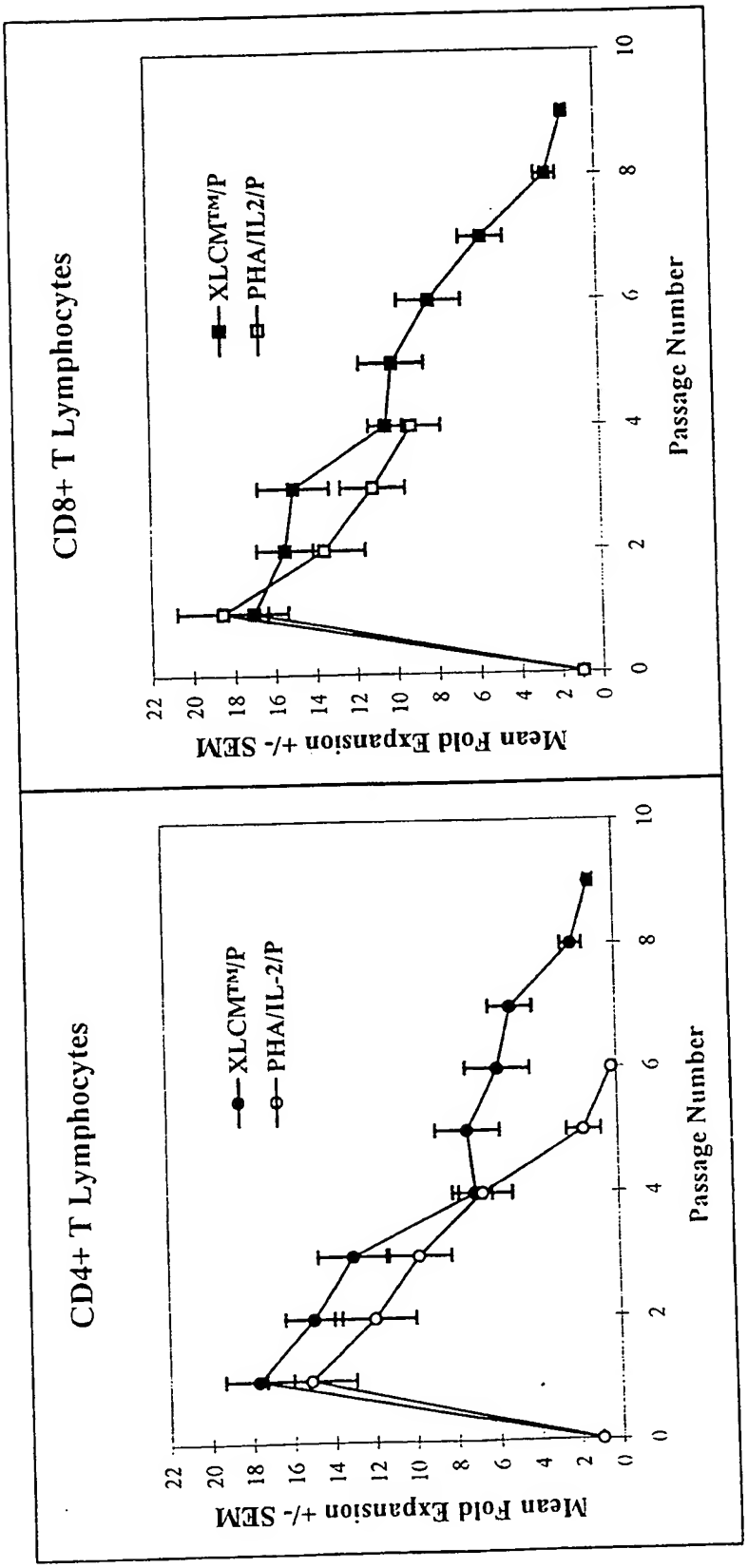


Figure 4: Expansion of T Cells from HIV+ Patients by XLCM/P Compared to PHA/IL-2/P

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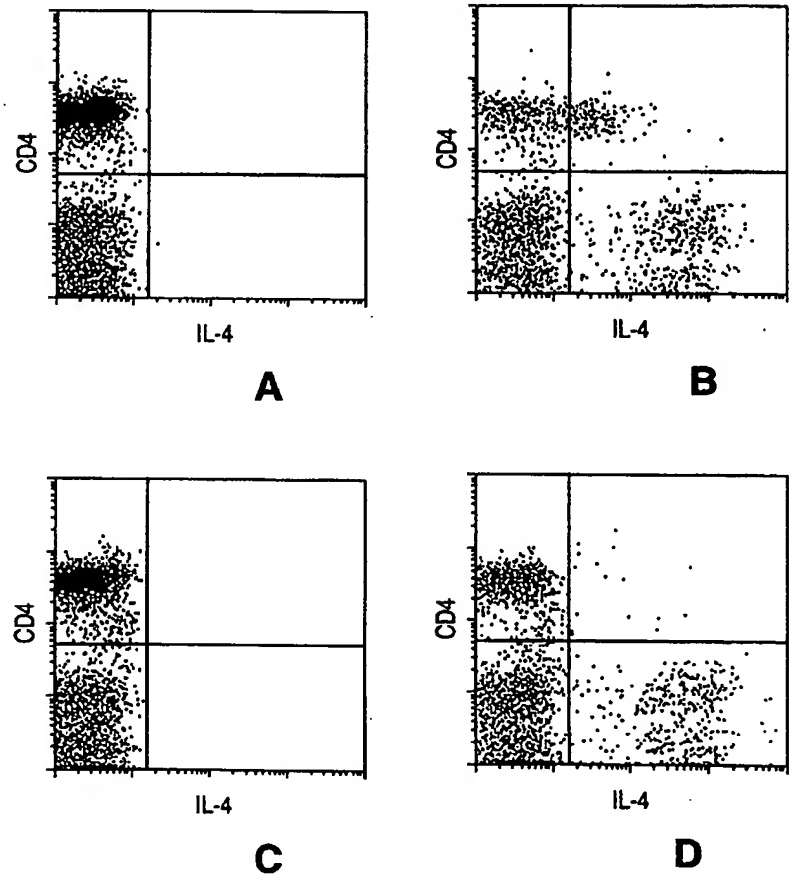
(54) Title: METHOD OF DIRECT SELECTION OF ANTIGEN–SPECIFIC T CELLS

(57) Abstract

The invention provides a method for convenient analysis and cell separation of antigen–specific T cells based on one or more products secreted by these cells in response to antigen stimulation. The T cells are provided with a capture moiety for the product, which can then be used directly as a label in some instances, or the bound product can be further labeled via label moieties that bind specifically to the product and that are labeled with traditional labeling materials such as fluorophores, radioactive isotopes, chromophores or magnetic particles. The labeled cells are then separated using standard cell sorting techniques based on these labels. Such techniques include flow cytometry, magnetic gradient separation, centrifugation, and the like.



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METHOD OF DIRECT SELECTION OF ANTIGEN-SPECIFIC T CELLS

TECHNICAL FIELD

The invention is in the field of analysis of cell populations and cell separation and the compositions obtained thereby. More particularly, the invention concerns analysis and separation of antigen-specific T cells based on primary labeling of cells with their secreted products through capture of these products by a specific binding partner for the product anchored or bound to the cell surface.

BACKGROUND ART

Numerous attempts have been made to analyze populations of cells and to separate cells based on the products which they produce. Such approaches to cell analysis and separation are especially useful in assessing those cells which are capable of secreting a desired product (the "product"), or which are relatively high secretors of the product. These methods include cloning in microtiter plates and analysis of the culture supernatant for product, cloning in agar and analysis by methods for identification of the product of the localized cells; the identification methods include, for example, plaque assays and western blotting. Most methods for analysis and selection of cells based upon product secretion involve physically isolating the cell, followed by incubation under conditions that allow product secretion, and screening of the cell locations to detect the cell or cell clones that produce the product. When cells are in suspension, after the cells have secreted the product, the product diffuses from the cell without leaving a marker to allow

identification of the cell from which it was secreted. Thus, secretor cells cannot be separated from non-secretor cells with these types of systems.

In other cases, both secretor and non-secretor cells can associate the product with the cell membrane. An example of this type of system are B cell derived cell lines producing monoclonal antibodies. It has been reported that these types of cell lines were separated by fluorescence activated cell sorting (FACS) and other methods reliant upon the presence of antibody cell surface markers. However, procedures that analyze and separate cells by markers that are naturally associated with the cell surface can not accurately identify and/or be used in the separation of secretor cells from non-secretor cells. In addition, systems such as these are not useful in identifying quantitative differences in secretor cells (i.e., low level secretors from high level secretors).

A method that has been used to overcome the problems associated with product diffusion from the cells has been to place the cell in a medium that inhibits the rate of diffusion from the cell. A typical method has been to immobilize the cell in a gel-like medium (agar), and then to screen the agar plates for product production using a system reliant upon blotting, for example Western blots. These systems are cumbersome and expensive if large numbers of cells are to be analyzed for properties of secretion, non-secretion, or amount of secretion.

Köhler et al. have described a negative-selection system in which mutants of a hybridoma line secreting IgM with anti-trinitrophenyl (anti-TNP) specificity were enriched by coupling the hapten (i.e., TNP) to the cell surface and incubating the cells in the presence of complement. In this way, cells secreting wild-type Ig were lysed, whereas cells secreting IgM with reduced lytic activity or not binding to TNP preferentially survived. Köhler and Schulman (1980) *Eur. J. Immunol.* 10:467-476.

More recently, a system has been described for labeling and separating cells based on secreted product. PCT/US93/10126. In this system, a specific binding partner for a secreted product is coupled to the surface of cells. The product is

secreted, released, and bound to the cell by the specific binding partner. Cells are then separated based on the degree to which they are labeled with the bound product.

Other systems allow the cells to secrete their products in the context of microdroplets of agarose gel which contain reagents that bind the secretion products, and encapsulation of the cells. Such methods have been disclosed in publications by Nir et al. (1990) *Applied and Environ. Microbiol.* 56:2870-2875; and Nir et al. (1991) *Applied and Environ. Microbiol.* 56:3861-3866. These methods are unsatisfactory for a variety of reasons. In the process of microencapsulation, statistical trapping of numbers of cells in the capsules occurs, resulting in either a high number of empty capsules when encapsulation occurs at low cell concentrations, or multiple cells per capsule when encapsulation occurs at high cell concentrations. Secreted product is trapped in the agarose drop by the capture antibody and detected by a second fluorochromated antibody. This process, while allowing for the detection and isolation of cells based on secreted product, is complicated, requires special equipment, and is not suited to all types of sorting methods.

In order to analyze and separate single cells or single cell clusters by this technique, large volumes must be handled to work with relatively small numbers of cells because of the numbers of empty capsules and because of the size of the microcapsules (50-100 μm). The large volume of droplets results in background problems using flow cytometry analysis and separation. In addition, the capsules do not allow separation using magnetic beads or panning for cell separation.

Various methods have been used to couple labels to cell surfaces where the label such as a fluorochrome is intended for direct detection. For example, hydrophobic linkers inserted into the cell membrane to couple fluorescent labels to cells have been described in PCT WO 90/02334, published 8 March 1990. Antibodies directed to HLA have also been used to bind labels to cell surfaces. Such binding results in a smaller dimension than the encapsulated droplets described above

and such cells can be conveniently used in standard separation procedures including flow cytometry and magnetic separations.

ELISpot assays and methods for intracellular cytokine staining have been used for enumeration and characterization of antigen-specific $CD4^+$ and $CD8^+$ T cells. Lalvani et al. (1997) *J. Exp. Med.* 186:859-865; and Waldrop et al. (1997) *J. Clin Invest.* 99:1739-1750. These methods can be quite useful for T-cell epitope mapping or for monitoring immunogenicity in vaccine trials, but they do not allow isolation of live antigen-specific T cells, e.g., for clinical trials of specific adoptive immunotherapy of cancer or infections. Kern et al. (1998) *Nat. Med.* 4:975-978; El Ghazali et al. (1993) *Curr. Opin Immunol.* 23:2740-2745; and Yee et al (1997) Curr. Opin. Immund. 9:702-708.

Soluble multivalent complexes of peptide-loaded major histocompatibility complex (MHC) molecules have been exploited recently to detect and also isolate antigen-specific T cells. Altman et al. (1996) *Science* 274:94-96; Dunbar et al. (1998) *Curr. Biol.* 8:413-416; Ogg et al. (1998) 279:2103-2106; Luxembourg et al. (1998) *Nat. Biotechnol.* 16:281-285; Murali-Krishna et al. (1998) *Immunity* 8:177-187; Gallimore et al. (1998) *J. Exp. Med.* 187:1383-1393; and Flynn et al. (1998) *Immunity* 8:683-691. These reagents are highly specific but the approach is limited to well defined combinations of antigenic peptides and restricting HLA alleles.

The immune system comprises two types of antigen-specific cells: B cells and T cells. T cells can be characterized phenotypically by the manner in which they recognize antigen, by their cell surface markers, and by their secreted products. Unlike B cells, which recognize soluble antigen, T cells recognize antigen only when the antigen is presented to them in the form of small fragments bound to major histocompatibility complex (MHC) molecules on the surface of another cell. Any cell expressing MHC molecules associated with antigen fragments on its surface can be regarded as an antigen-presenting cell (APC). In most situations, however, more than the mere display of an MHC-bound antigen fragment on a cell surface is

required to activate a T lymphocyte. In addition to the signal delivered via the T cell receptor (TCR) engaged by MHC molecule plus antigen, the T cell must also receive co-stimulatory signals from the APC. Typically APCs are dendritic cells, macrophages or activated B lymphocytes.

T cells express distinctive membrane molecules. Included among these are the T cell antigen receptor (TCR), which appears on the cell surface in association with CD3; and accessory molecules such as CD5, CD28 and CD45R. Subpopulations of T cells can be distinguished by the presence of additional membrane molecules. Thus, for example, T cells that express CD4 recognize antigen associated with class II MHC molecules and generally function as helper cells, while T cells that express CD8 recognize antigen associated with class I MHC molecules and generally function as cytotoxic cells. The $CD4^+$ subpopulation of T cells can be categorized further into at least two subsets on the basis of the types of cytokines secreted by the cell. Thus, while both subsets secrete IL-3 and GM-CSF, TH1 cells generally secrete IL-2, IFN-γ, and TNF-α, whereas TH2 cells generally secrete IL-4, IL-5, IL-10, and IL-13.

Minor changes in the peptide bound to the MHC molecule can not affect the affinity of the peptide-MHC molecule interaction, yet they can generate partial signals that lead to a halfway response characterized by proliferation and secretion of only a fraction of the cytokines produced during a full T cell response. Some modified peptides can even block proliferation and cytokine secretion altogether and induce a state of T cell anergy or unresponsiveness. There are thus three different types of peptides: agonist (those that stimulate a full response), partial agonist (those that stimulate a partial response) and antagonist (those that induce unresponsiveness). When a single APC presents a mixture of an agonist and an antagonist on its surface, the negative effect of the latter can overcome the positive effect of the former, even if the antagonist is present in much smaller amounts than the agonist. Some viruses seem to use mutations in their proteins to produce antagonist peptides capable of

suppressing the activity of the T cell clones that recognize agonist peptides derived from the original wild-type virus.

Secretion by a T cell of a particular cytokine is generally associated with a particular function. For example, differences in the cytokines secreted by the TH1 and TH2 subsets of $CD4^+$ T cells are believed to reflect different biological functions of these two subsets. The TH1 subset is responsible for classical cell-mediated functions such as delayed-type hypersensitivity and activation of cytotoxic T cells, whereas the TH2 subset functions more effectively as a helper for B-cell activation. The TH1 subset can be particularly suited to respond to viral infections and intracellular pathogens because it secretes IL-2 and IFN-γ, which activate cytotoxic T cells. The TH2 subset can be more suited to respond to extracellular bacteria and helminthic parasites and can mediate allergic reactions, since IL-4 and IL-5 are known to induce IgE production and eosinophil activation, respectively. There is also considerable evidence suggesting that preferential activation of TH1 cells plays a central role in the pathogenesis of a number of autoimmune diseases. Secretion of IL-10 by TH2 cells is thought to suppress, in an indirect manner, cytokine production by TH1 cells, and, accordingly, has a general immunosuppressive effect. A shift in the TH1/TH2 balance can result in an allergic response, for example, or, in an increased cytotoxic T cell response.

The changes initiated by the TCR in the first few minutes to hours of activation lead to transition of the cell from the G0 to G1 phase of the cell cycle. Several hours after stimulation of the T cell begins to express IL-2 and high-affinity IL-2 receptor. *IL2* gene expression is effected by a set of transcription factors that are activated by the converging signaling pathways triggered by the ligation of TCR, CD28 and possibly other T cell surface molecules.

The transcription factors also induce expression of the CD25 gene, which encodes the α-subunit of the high-affinity IL-2 receptor. The interaction of IL-2 with the high-affinity receptor initiates signaling pathways that cause the T cell to transit

from the G1 to the S phase of the cell cycle and progress to cell division. The signaling pathways control the expression and activity of several key proteins necessary for cell division. Some of these are also activated directly by TCR- and CD28-dependent signals while others are energized only by signals provided via the IL-2 receptor.

The stimulated T cell undergoes a sequence of phenotypic changes beginning with its progression from the resting state to mitosis and later to differentiation into effector and memory cells. Among the earliest (immediate) changes, observable within 15-30 minutes of stimulation, are the expression of genes encoding transcription factors such as c-Fos, NF-AT, c-Myc and NF-κB, protein kinases such as Jak-3 and protein phosphatases such as Pac-1. The subsequent early changes, occurring within several hours of stimulation, mark the beginning of the expression of genes encoding activation antigens. These include several cytokines (IL-2 and others), IL-2 receptor subunit α (CD25), insulin receptor, transferrin receptor and several other surface molecules such as CD 26, CD30, CD54, CD69 and CD70.

Activation antigens reach a maximum level of expression just before the first division, 24 hours after stimulation. During this period the level of expression of several other molecules already expressed on resting T cells increases. At a later point, some days after activation commenced, late activation antigens become expressed on the T cells. These include MHC class II molecules and several members of the β1 integrin family. Expression of late activation antigens marks the differentiation of the activated cell into effector or memory T cells.

T cells play important roles in autoimmunity, inflammation, cytotoxicity, graft rejection, allergy, delayed-type hypersensitivity, IgE-mediated hypersensitivity, and modulation of the humoral response. Disease states can result from the activation of self-reactive T cells, from the activation of T cells that provoke allergic reactions, or from the activation of autoreactive T cells following certain bacterial and parasitic infections, which can produce antigens that mimic human protein, rendering these

protein "autoantigens." These diseases include, for example, the autoimmune diseases, autoimmune disorders that occur as a secondary event to infection with certain bacteria or parasites, T cell-mediated allergies, and certain skin diseases such as psoriasis and vasculitis. Furthermore, undesired rejection of a foreign antigen can result in graft rejection or even infertility, and such rejection can be due to activation of specific T lymphocyte populations. Pathological conditions can also arise from an inadequate T cell response to a tumor or a viral infection. In these cases, it would be desirable to increase an antigen-specific T cell response in order to reduce or eliminate the tumor or to eradicate an infection.

Autoimmune diseases have a variety of causes. For instance, autoimmune reactions can be provoked by injury or immunization with collagen, by superantigens, by genetic factors, or errors in immune regulation. Superantigens are polyclonal activators that can, among other things, stimulate clones previously anergized by an encounter with an autoantigen or clones that ignored the potential autoantigens because of their low expression or availability. Certain autoimmune disease are caused mainly by autoantibodies, others are T cell-mediated. Autoreactive T cells cause tissue damage in a number of autoimmune diseases including rheumatoid arthritis and multiple sclerosis.

In the treatment of autoimmune disorders, nonspecific immune suppressive agents have been used to slow the disease; these therapies often cause a general immunosuppression by randomly killing or inhibiting immunocompetent cells. Attempts to treat autoimmune disorders by modulating the activity of autoreactive T cells have included immunization with TCR peptides, treatment with interferon-β (IFN-β) and T lymphocyte vaccination. Ebers (1994) *Lancet* 343:275-278; Hohlfeld (1997) *Brain* 120:865-916; and Hafler et al. (1992) *Clin. Immunol. Immunopathol.* 62:307-313.

The development of allergic sensitization, contact sensitivity and inflammation is dependent on activation and stimulation of T cells that exhibit pro-

allergic functions. Allergen-specific T cells are believed to play an important role in the pathophysiology of atopic allergies. Elimination or suppression of allergen-specific T cells could help ameliorate allergic diseases caused by such T cells.

In the initial phase of an allergic reaction, antigen (allergen) enters the body, is picked up by APCs, displayed by them in the context of class II MHC molecules and recognized by helper T cell precursors. These are stimulated to proliferate and differentiate mainly into TH2 cells, which help B lymphocytes differentiate into antibody-producing plasma cells. As in any other antibody-mediated response, the B cells that receive specific help from TH cells are those that recognized the allergen via their surface receptors. Some of the cytokines produced by the TH2 cells, especially IL-4 and IL-13, stimulate the B cells to effect an immunoglobulin isotype switch and to produce IgE antibodies. The antibodies bind to high-affinity Fc receptors on the surface of mast cells in the connective tissue and mucosa, as well as to those on the surface of basophils in the circulation and mucosa and initiate the manifestations of allergic reaction.

Allograft rejection is caused principally by a cell-mediated immune response to alloantigens (primarily MHC molecules) expressed on cells of the graft. Analysis of the T lymphocyte subpopulations involved in allograft rejection has implicated both $CD4^+$ and $CD8^+$ populations. TH1 cells initiate the inflammatory reaction of delayed-type hypersensitivity, leading to the recruitment of monocytes and macrophages into the graft. Natural kill (NK) cells, presumably alerted by the absence in the graft of MHC molecules present in the recipient, can also attack the graft in the early phases of the response. Neutrophils are mainly responsible for clearing the wound or removing damaged cells and cellular debris in the late phase of the allograft reaction.

Most immunosuppressive treatments developed have the disadvantage of being non-specific; that is, they result in generalized immunosuppression, which places the recipient at increased risk for infection. Immunosuppressive agents

employed to prevent organ rejection include mitotic inhibitors such as azathioprine, cyclophosphamide and methotrexate; corticosteroids; and drugs, such as cyclosporin, FK506 and rapamycin, which inhibit the transcription of the genes encoding IL-2 and the high-affinity receptor for IL-2.

In the treatment of cancers, cellular immunotherapy has been employed as an alternative, or an adjunct to, conventional therapies such as chemotherapy and radiation therapy. For example, cytotoxic T lymphocyte (CTL) responses can be directed against antigens specifically or preferentially presented by tumor cells. Following activation with T cell cytokines in the presence of appropriately presented tumor antigen, tumor infiltrating lymphocytes (TILs) proliferate in culture and acquire potent anti-tumor cytolytic properties. Weidmann et al. (1994) *Cancer Immunol. Immunother.* 39:1-14.

The introduction into a cancer patient of *in vitro* activated lymphocyte populations has yielded some success. Adoptive immunotherapy, the infusion of immunologically active cells into a cancer patient in order to effect tumor regression, has been an attractive approach to cancer therapy for several decades. Two general approaches have been pursued. In the first, donor cells are collected that are either naturally reactive against the host's tumor, based on differences in the expression of histocompatibility antigens, or made to be reactive using a variety of "immunizing" techniques. These activated donor cells are then transfused to a tumor-bearing host. In the second general approach, lymphocytes from a cancer patient are collected, activated *ex vivo* against the tumor and then reinfused into the patient. Triozzi (1993) *Stem Cells* 11:204-211; and Sussman et al. (1994) *Annals Surg. Oncol.* 1:296.

Current methods of cancer treatment are relatively non-selective. Surgery removes the diseased tissue, radiotherapy shrinks solid tumors and chemotherapy kills rapidly dividing cells. Systemic delivery of chemotherapeutic agents, in particular, results in numerous side effects, in some cases severe enough to preclude the use of potentially effective drugs.

Viral diseases are also candidates for immunotherapy. Heslop et al. (1996) *Nature Med.* 2:551-555. Immunological responses to viral pathogens are sometimes ineffective in eradicating or sufficiently depleting the virus. Furthermore, the highly mutable nature of certain viruses, such as human immunodeficiency virus, allows them to evade the immune system.

Clearly, there is a need to identify, analyze and enrich populations of T cells involved in the above-mentioned pathologies. Currently, several methods for analysis and for enrichment of antigen-specific and/or cytokine-secreting T cells exist. Enrichment of antigen-specific T cells can be achieved using selective culturing techniques to obtain T cell lines and T cell clones. These techniques generally involve culturing the T cells *in vitro* over a period of several weeks and using rather cumbersome methods to select lines or clones exhibiting the desired phenotype, such as cytokine secretion. Other attempts to detect and enrich for antigen-specific T cells have employed defined multimeric MHC-antigen and MHC - peptide complexes. U.S. Patent No. 5,635,363. For such a technique to be successful, however, MHC-antigen complexes of the correct MHC allotype are required, and the selection is limited to antigen specificity, i.e., no selection for cytokine secretion is afforded by this technique.

Intracellular cytokine staining after antigen activation, followed by FACS analysis, is the method used to obtain information regarding the antigen specificity and kinetics of cytokine production. Waldrop et al. (1997) *J. Clin. Invest.* 99:1739-1750. This method is useful for analysis only, since the cells are not viable after this procedure. Similarly, cytokine ELISPOT assays are useful for analysis only. Miyahira et al. (1995) *J. Immunol. Met.* 181:45-54; and Lalvani et al. (1997) *J. Exp. Med.* 186:859-865. In these assays, secreted cytokines are trapped in a surrounding matrix for analysis, but there is no mechanism for identifying and retrieving the cell which secreted the cytokine. The gel microdrop technology is not suited to

processing large numbers of cells such as would be necessary for treatment of the above-mentioned indications.

It is apparent from the foregoing discussion that there is a need for reliable techniques for analyzing and separating populations of T cells, based on secreted product, for a number of therapeutic and diagnostic purposes. The present invention addresses this need by providing methods for analyzing, separating and enriching populations of antigen-specific T cells.

DISCLOSURE OF THE INVENTION

The invention provides a method for convenient analysis and cell separation of antigen-specific T cells based on one or more products secreted by these cells in response to antigen stimulation. The T cells are provided with a capture moiety specific for the product (or, "specific binding partner"), which can then be used directly as a label. The binding of the product to the capture moiety results in a "captured product." Alternatively, the cells are bound to the product via the capture moiety and can be further labeled via label moieties that bind specifically to the product and that are, in turn, labeled either directly or indirectly with traditional labeling materials such as fluorophores, radioactive isotopes, chromophores or magnetic particles.

The labeled cells can then be separated using standard cell sorting techniques based on these labels. Such techniques include, but are not limited to, flow cytometry, FACS, high gradient magnetic gradient separation, centrifugation.

Thus, in one aspect, the invention encompasses a method to stimulate and separate antigen-specific T cells from a population of cells according to a product secreted and released by the antigen specific T cells in response to the stimulation. The method comprises stimulating a mixture of cells containing T cells with antigen, and effecting a separation of antigen-stimulated cells according to the degree to which they are labeled with the product. Antigen stimulation is achieved by exposing

the cells to at least one antigen under conditions effective to elicit antigen-specific stimulation of at least one T cell. Labeling with the product is achieved by modifying the surface of the cells to contain at least one capture moiety, culturing the cells under conditions in which the product is secreted, released and specifically bound ("captured" or "entrapped") to said capture moiety; and labeling the captured product with a label moiety, where the labeled cells are not lysed as part of the labeling procedure or as part of the separation procedure.

Another aspect of the invention is a composition of matter containing antigen-specific T cells capable of capturing a product secreted and released by these cells in response to antigen stimulation, where the surface of the cells is modified to contain a capture moiety for the product. The captured product can be separately labeled by a label moiety.

Still another aspect of the invention is antigen-specific T cells and progeny thereof separated by the above-described method.

Yet another aspect of the invention is a method to label antigen-specific T cells with a product secreted and released by the cells in response to antigen stimulation, by modifying the surface of these cells to contain a specific binding partner for the product coupled to the cell surface, and culturing the cells under conditions wherein the product is secreted and released.

An additional aspect of the invention is a method of analyzing a population of antigen-specific T cells to determine the proportion of cells that secrete an amount of product relative to other cells in the population, where the product is secreted in response to antigen stimulation. The method comprises labeling the cells by the above-described method, further labeling the cells with a second label that does not label the captured product, and detecting the amount of product label relative to the second cell label. Such methods are useful, for example, in assessing the immune status of an individual.

A further aspect of the invention is methods for use of T cell populations enriched in antigen-specific T cells. The methods comprise administering to an individual in need of treatment a composition comprising a T cell population enriched in antigen-specific T cells. Such methods are useful to treat a variety of pathological conditions, including cancer, allergies, immunodeficiencies, autoimmune disorders, and viral diseases.

Yet another aspect of the invention is a kit for use in separation of antigen-specific T cells from a mixed population comprising effector cells. The kit can contain a physiologically acceptable medium which can be of varying degrees of viscosity up to a gel-like consistency, a product capture system comprising anchor and capture moieties; a label system for detecting the captured product; and instructions for use of the reagents, all packaged in appropriate containers. Optionally, the kit further comprises a magnetic labeling system and/or one or more biological modifiers.

Still another aspect of the invention is a kit for use in the detection/separation of antigen-specific T cells that secrete a desired product in response to antigen stimulation, the kit comprising a product capture system comprising anchor and capture moieties; a label system for detecting the captured product; and instructions for use of the reagents, all packaged in appropriate containers. Optionally, the kit further comprises a magnetic labeling system, and/or antigen, and/or one or more biological modifiers.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 A-P are FACS plots showing analysis of cells subjected to the separation protocol described in Example 1. A-H show analysis of control cells cultured with no peptide; I-P show analysis of peptide-stimulated cells. A, C, I, and K show scatter properties of the starting cell population (A and I) and the enriched cell population (C and K). B, D, J and L show profiles of PI versus PE staining of the

starting cell population (B and J) and the enriched cell population (D and L). Plots E-H and M-P show FITC-labeled anti-CD8 versus PE-labeled anti-IFN-γ staining of the starting cell population (E and M), the first negative population (F and N), the second negative population (G and O) and the enriched cell population (H and P).

Figures 2A-N are FACS plots showing analysis of cells subjected to the separation protocol described in Example 2. A-G show analysis of control cells cultured with no peptide; N-R show analysis of peptide-stimulated cells. A-D and H-K show FITC-labeled anti-CD8 versus PE-labeled anti-IFN-γ staining of the starting cell population (A and J), the first negative population (B and I), the second negative population (C and J) and the enriched cell population (D and K). F and M show staining for Vβ17TCR of the enriched cell population.

Figure 3 is a series of dot plots showing IFN-γ-secretion-based enrichment and detection of live antigen-specific $CD4^+$ and $CD8^+$ T cells. Dot plots show CD8-Cy5 vs. anti IFN-γ-PE (A-D) or CD4-Cy5 vs. anti IFN-γ-PE (E-L) staining of PBMC from healthy adult donors stimulated with (A,B) or without (C,D) the HLA-A0201-restricted FLU 58-66 peptide, a purified influenza A virus preparation (with (E,F) without (G,H)) and rTT.C (with (I,J) without (K,L)) before (A,C,E,G,I,K) and after (B,D,F,H,J,L) magnetic enrichment of IFN-γ-secreting cells. Live lymphocytes were gated according to light-scatter properties and propidium iodide exclusion.

Figure 4 is a series of dot plots showing a phenotypic analysis of enriched Flu 58-66 peptide-specific $CD8^+$ T cells. Enriched IFN-γ-secreting $CD8^+$ T cells from FLU 58-66 peptide-stimulated PBMC (A,B,E,F) and, for control, from non-stimulated PBMC (C,D,G,H) were stained with anti IFN-γ-PE and counterstained with FITC-conjugated antibodies against CD27, CD28, CD57 and the TCR Vβ17 chain. Light-scatter properties, propidium iodide and CD8-Cy5 staining were used for gating of live $CD8^+$ T cells.

Figure 5 is a graph depicting cytolytic activity of enriched and expanded Flu 58-66 peptide-specific T cells. IFN-γ-secreting $CD8^+$ T cells from FLU 58-66

peptide-stimulated PBMC were expanded for 18 days in tissue culture in the presence of IL-2 and then assayed for CTL activity assay. The diagram shows the percentage of lysed HLA-A2.1+ T2 cells pulsed with either Flu 58-66 peptide or the negative control peptide Melan A/MART 1 27-35.

Figure 6 is a series of dot plots depicting the isolation and detection of TT-specific IL-4-secreting CD4+ T cells. Dot plots show CD4-Cy5 vs. anti IL-4-PE staining of PBMC from healthy adult donors stimulated with (A,C) or without (B,D) magnetic enrichment of IL-4-secreting cells. Live lymphocytes were gated according to light-scatter properties and propidium iodide exclusion.

MODES FOR CARRYING OUT THE INVENTION

The present invention provides methods for detecting, analyzing and separating antigen-stimulated T cells on the basis of secreted product, where the product is secreted as a result of antigen stimulation. The methods are based on capture and relocation to the cell surface of the secreted product. The captured product permits the cell to be detected, analyzed and, if desired, sorted, according to the presence, absence or amount of the product present. The means of capture comprises a product-specific binding partner ("capture moiety") anchored to the cell surface by a means suitable for the cell to be sorted.

The approach presented here combines, *inter alia*, the following advantages: (a) it permits rapid isolation, enumeration, phenotyping and expansion of live antigen-specific T lymphocytes without the need of cyclical activation of T cells with antigen and APCs; (b) it is generally applicable for isolation of T cells reactive to APCs that have been pulsed with synthetic peptides, native proteins, cell extracts, nonviable pathogens, transduced with retroviral vectors, infected with recombinant viral vectors, transfected with RNA or DNA, etc.; (c) it can be used for the isolation of both $CD4^+$ antigen-specific Th cells and $CD8^+$ antigen-specific CTLs; and (d) it enables selective isolation of antigen specific T cells with particular cytokine-

mediated effector functions, e.g., of antigen-specific Th1-, Th2-, or Th3-like lymphocytes.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (D.M. Weir & C.C. Blackwell, eds.); "Gene Transfer Vectors for Mammalian Cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987, and periodic updates); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994); and "Current Protocols in Immunology" (J.E. Coligan et al., eds., 1991).

Cell sorting and cell analysis methods are known in the art and are described in, for example, *The Handbook of Experimental Immunology*, Volumes 1 to 4, (D.N. Weir, editor) and *Flow Cytometry and Cell Sorting* (A. Radbruch, editor, Springer Verlag, 1992).

As used herein, a "specific binding partner" or "capture moiety" intends a member of a pair of molecules (a "specific binding pair") that interact by means of specific non-covalent interactions that depend on the three-dimensional structures of the molecules involved. A "label moiety" is detectable, either directly or indirectly. When the capture moiety is an antibody, it can be referred to as the "capture antibody" or "catch antibody." The capture moieties are those which attach both to the cell, either directly or indirectly, and the product. The label moieties are those which attach to the product and can be directly or indirectly labeled.

As used herein, the term "antibody" is intended to include polyclonal and monoclonal antibodies, chimeric antibodies, haptens and antibody fragments, and

molecules which are antibody equivalents in that they specifically bind to an epitope on the product antigen. The term "antibody" includes polyclonal and monoclonal antibodies of any isotype (IgA, IgG, IgE, IgD, IgM), or an antigen-binding portion thereof, including, but not limited to, F(ab) and Fv fragments such as sc Fv, single chain antibodies, chimeric antibodies, humanized antibodies, and a Fab expression library. Antibodies can also be immobilized for instance on a polymer or a particle.

"Bispecific antibody" and "bispecific antibodies," also known as bifunctional antibodies, intends antibodies that recognize two different antigens by virtue of possessing at least one first antigen combining site specific for a first antigen or hapten, and at least one second antigen combining site specific for a second antigen or hapten. Such antibodies can be produced by recombinant DNA methods or include, but are not limited to, antibodies chemically by methods known in the art. Chemically created bispecific antibodies that have been reduced and reformed so as to retain their bivalent characteristics and antibodies that have been chemically coupled so that they have at least two antigen recognition sites for each antigen. Bispecific antibodies include all antibodies or conjugates of antibodies, or polymeric forms of antibodies which are capable of recognizing two different antigens. The label moiety can be a fluorochromated antiproduct antibody, which can include, but is not limited to, magnetic bead conjugated, colloidal bead conjugated, FITC, Phycoerythrin, PerCP, AMCA, fluorescent particle or liposome conjugated antibodies. Alternatively the label moiety can be any suitable label including but not limited to those described herein. Bispecific antibodies include antibodies that have been reduced and reformed so as to retain their bivalent characteristics and to antibodies that have been chemically coupled so that they can have several antigen recognition sites for each antigen.

As used herein the term "effector cell population" intends a cell population which comprises at least one T cell. An effector cell population can be obtained from a starting cell population from which antigen-specific T cells are enriched.

The terms "cell," and "cells," and "cell population," used interchangeably, intend one or more mammalian cells. The term includes progeny of a cell or cell population. Those skilled in the art will recognize that "cells" include progeny of a single cell, and the progeny can not necessarily be completely identical (in morphology or of total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation and/or change.

The terms "T lymphocyte," "T cell," "T cells," and "T cell population," used interchangeably, intends a cell or cells which display on their surface one or more antigens characteristic of T cells, such as, for example, CD2 and CD3. The term includes progeny of a T cell or T cell population. A "T lymphocyte" or "T cell" is a cell which expresses CD3 on its cell surface and a T cell antigen receptor (TCR) capable of recognizing antigen when displayed on the surface of autologous cells, or any antigen-presenting matrix, together with one or more MHC molecules or, one or more non-classical MHC molecules. The term "T cells" as used herein denotes any T cells known in the art, for instance, lymphocytes that are phenotypically $CD3^+$, i.e., express CD3 on the cell surface, typically detected using an anti-CD3 monoclonal antibody in combination with a suitable labeling technique. The T cells enriched by the methods of this invention are generally $CD3^+$. The T cells enriched by the methods of this invention are also generally, although not necessarily, positive for CD4, CD8, or both.

The term "substantially enriched" as used herein, indicates that a cell population is at least about 50-fold, more preferably at least about 500-fold, and even more preferably at least about 5000-fold or more enriched from an original mixed cell population comprising the desired cell population.

The term "antigen-presenting matrix," as used herein, intends a molecule or molecules which can present antigen in such a way that the antigen can be bound by a T cell antigen receptor on the surface of a T cell. An antigen-presenting matrix can be on the surface of an antigen-presenting cell (APC), on a vesicle preparation of an

APC, or can be in the form of a synthetic matrix on a bead or a plate. The term "antigen presenting cell", as used herein, intends any cell which presents on its surface an antigen in association with a MHC or portion thereof, or, one or more non-classical MHC molecules, or a portion thereof.

The term "autogeneic," "autologous," or, "self," as used herein, indicates the origin of a cell. Thus, a cell is autogeneic if the cell was derived from an individual (the "donor") or a genetically identical individual and is to be readministered to the individual. An autogeneic cell can also be a progeny of an autogeneic cell. The term also indicates that cells of different cell types are derived from the same donor or genetically identical donors. Thus, an effector cell and an antigen presenting cell are said to be autogeneic if they were derived from the same donor or from an individual genetically identical to the donor, or if they are progeny of cells derived from the same donor or from an individual genetically identical to the donor.

Similarly, the term "allogeneic," or "non-self," as used herein, indicates the origin of a cell. Thus, a cell or the progeny thereof is allogeneic if the cell was derived from an individual not genetically identical to the recipient to whom it is administered. The term relates to non-identity in expressed MHC molecules. The term also indicates that cells of different cell types are derived from genetically non-identical donors, or if they are progeny of cells derived from genetically non-identical donors. For example, an APC is said to be allogeneic to an effector cell if they are derived from genetically non-identical donors.

A "disease or condition related to a population of antigen-specific T cells" is one which can be related to a population of antigen-specific T cells or lack of adequate numbers thereof, and includes, for example, autoimmune diseases in which antigen-specific T cells are primarily responsible for the pathogenesis of the disease; cancers, in which cancerous cell growth is not adequately controlled by tumor-specific cytotoxic T cells; viral diseases, in which virus-infected cells are not lysed by cytotoxic T cells; allergies, in which T cells specific for allergens mediate undesired

effects; immunodeficiencies, in which inadequate numbers of T cells are present in an individual due to either infection (such as HIV) or congenitally (such as DiGeorge syndrome). It is also one in which antigen-specific T cells modulate or regulate the activity of another cell or cell population which is primarily responsible for a disease state; it is also one in which the presence of a population of antigen-specific T cells is not the primary cause of the disease, but which plays a key role in the pathogenesis of the disease; it is also one in which a population of antigen-specific T cells mediates an undesired rejection of a foreign antigen.

An "individual" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, humans farm animals, sport animals, and pets.

An "effective amount" is an amount sufficient to effect beneficial or desired clinical results. An effective amount can be administered in one or more administrations. For purposes of this invention, an effective amount of antigen-specific T cells is an amount that is sufficient to diagnose, palliate, ameliorate, stabilize, reverse, slow or delay the progression of the disease state.

As used herein, "treatment" is an approach for obtaining beneficial or desired clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, preventing spread (i.e., metastasis) of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment.

"Palliating" a disease means that the extent and/or undesirable clinical manifestations of a disease state are lessened and/or time course of the progression is slowed or lengthened, as compared to not administering enriched T cell populations of the present invention.

The present invention provides methods for obtaining a cell population enriched in antigen-specific T cells which secrete a product, where the product is secreted as a result of antigen stimulation. The methods generally involve obtaining a mixed population of cells comprising T cells; exposing the cell population to at least one antigen under conditions effective to elicit antigen-specific stimulation of at least one T cell; modifying the surface of said mixed population to contain attached thereto a specific binding partner for the product; allowing expression of at least one product by the stimulated T cells, wherein the product is secreted in response to the stimulation; allowing binding of the product to a capture moiety coupled to the surface of the cell to form a cell bound capture moiety-product complex, thereby labeling the cells; and separating the stimulated T cells according to the degree to which they are labeled with said product.

Of course, modification of the cell surface with a specific binding partner can be carried out before, during, or after antigen stimulation.

Antigen presenting matrices and effector cell populations

The present invention provides methods for obtaining a cell population enriched in antigen-specific T cells which secrete a product in response to antigen stimulation. The methods comprise obtaining a mixed population of cells (i.e., an "effector cell population"), and exposing the cell population to at least one antigen. The mixed cell population can be obtained by any method known in the art and is preferably enriched for T cells. Exposure to antigen can be achieved using antigen-presenting matrices, which can be on the surface of antigen-presenting cells (APC's). Antigen-presenting matrices and effector cells can be obtained from a variety of sources. The mixed population of cells can be stimulated by antigen *in vitro* or *in vivo*, or modified in any of a variety of ways, for example, chemically or genetically modified.

Antigen presenting matrices

The T cell populations which are subjected to the methods of the present invention are exposed to at least one antigen under conditions effective to elicit antigen-specific stimulation. A T cell which is stimulated by the at least one antigen is said to be antigen specific, i.e., it displays on its cell surface an antigen receptor which specifically recognizes and binds to an antigen in association with a molecule capable of presenting antigen, such as a classical or non-classical MHC molecule or a portion thereof, on an antigen-presenting matrix, for example, a synthetic antigen-presenting matrix or one that is present on the surface of an APC.

The antigen-presenting molecule can be an MHC molecule, which can be class I or class II or, a non-classical MHC molecule such as CD1; an MHC epitope; a fusion protein comprising an MHC epitope; or a synthetic MHC epitope. The nature of the antigen-presenting molecule is not critical, so long as it is capable of presenting antigen to an effector cell. Methods of preparing MHC epitopes are known in the art.

Antigen-presenting matrices include those on the surface of an APC as well as synthetic antigen-presenting matrices. APCs suitable for use in the present invention are capable of presenting exogenous peptide or protein or endogenous antigen to T cells in association with an antigen-presenting molecule, such as an MHC molecule. APCs include, but are not limited to, macrophages, dendritic cells, CD40-activated B cells, antigen-specific B cells, tumor cells, virus-infected cells and genetically modified cells.

APCs can be obtained from a variety of sources, including but not limited to, peripheral blood mononuclear cells (PBMC), whole blood or fractions thereof containing mixed populations, spleen cells, bone marrow cells, tumor infiltrating lymphocytes, cells obtained by leukapheresis, lymph nodes, e.g., lymph nodes draining from a tumor. Suitable donors include an immunized donor, a non-immunized (naïve) donor, treated or untreated donors. A "treated" donor is one that has been exposed to one or more biological modifiers. An "untreated" donor has not

been exposed to one or more biological modifiers. APC's can also be treated *in vitro* with one or more biological modifiers.

The APCs are generally alive but can also be irradiated, mitomycin C treated, attenuated, or chemically fixed. Further, the APCs need not be whole cells. Instead, vesicle preparations of APCs can be used.

APCs can be genetically modified, i.e., transfected with a recombinant polynucleotide construct such that they express a polypeptide or an RNA molecule which they would not normally express or would normally express at lower levels. Examples of polynucleotides include, but are not limited to, those which encode an MHC molecule; a co-stimulatory molecule such as B7; or an antigen. For example, expression of a polynucleotide encoding an MHC molecule under transcriptional control of a strong promoter such as the CMV promoter, can result in high level expression of the MHC molecule on the cell surface, thus increasing the density of antigen presentation. Alternatively, an APC can be transfected with a polynucleotide construct comprising a polynucleotide encoding an antigen under transcriptional control of a strong promoter such as the CMV promoter such that the antigen is expressed on the cell surface together with an MHC molecule.

The nucleotide sequence encoding a polypeptide is operably linked to control sequences for transcription and translation. A control sequence is "operably linked" to a coding sequence if the control sequence regulates transcription or translation. Any method in the art can be used for the transformation, or insertion, of an exogenous polynucleotide into an APC, for example, lipofection, transduction, infection or electroporation, using either purified DNA, viral vectors, or DNA or RNA viruses. The exogenous polynucleotide can be maintained as a non-integrated vector, for example, a plasmid, or, can be integrated into the host cell genome.

Cells which do not normally function *in vivo* in mammals as APCs can be modified to function as APCs. A wide variety of cells can function as APCs when appropriately modified. Examples of such cells are insect cells, for example

Drosophila or *Spodoptera*; foster cells, such as the human cell line T2, which bears a mutation in its antigen presenting pathway that restricts the association of endogenous peptides with cell surface MHC class I molecules. Zweerink et al. (1993) *J. Immunol.* 150:1763-1771. For example, expression vectors which direct the synthesis of one or more antigen-presenting polypeptides, such as MHC molecules, and, optionally, accessory molecules such as B7, can be introduced into these cells to effect the expression on the surface of these cells antigen presentation molecules and, optionally, accessory molecules or functional portions thereof. Alternatively, antigen-presenting polypeptides and accessory molecules which can insert themselves into the cell membrane can be used. For example, glycosyl-phosphotidylinositol (GPI)-modified polypeptides can insert themselves into the membranes of cells. Medof et al. *J. Exp. Med.* 160:1558-1578; and Huang et al. *Immunity* 1:607-613. Accessory molecules include, but are not limited to, co-stimulatory antibodies such as antibodies specific for CD28, CD80, or CD86; costimulatory molecules, including, but not limited to, B7.1 and B7.2; adhesion molecules such as ICAM-1 and LFA-3; and survival molecules such as Fas ligand and CD70. See, for example, PCT Publication No. WO 97/46256.

Alternatively, a synthetic antigen-presenting matrix can be used to present antigen to effector cells. A synthetic matrix can include an antigen presenting molecule, preferably an MHC Class I or MHC Class II molecule, immobilized on a solid support, for example, beads or plates. Accessory molecules can be present, which can be co-immobilized or soluble, the molecules including, but not limited to, co-stimulatory antibodies such as antibodies specific for CD28, CD80, or CD86; costimulatory molecules, including, but not limited to, B7.1 and B7.2; adhesion molecules such as ICAM-1 and LFA-3; and survival molecules such as Fas ligand and CD70. Portions of accessory molecules can also be used, as long as their function is maintained. Solid supports include metals or plastics, porous materials,

microbeads, microtiter plates, red blood cells, and liposomes. See, for example, PCT Publication No. WO 97/46256; and WO 97/35035.

Methods for determining whether an antigen-presenting matrix, whether it is on a cell surface or on a synthetic support, is capable of presenting antigen to an effector cell, are known in the art and include, for example, ^{3}H-thymidine uptake by effector cells, cytokine production by effector cells, and cytolytic ^{51}Cr-release assays.

Effector cell populations

Antigen-specific T cells can be isolated from an effector cell population, i.e., a population of hematopoietic cells, preferably enriched for T cells. The effector cell population is a starting population from which antigen-specific T cells are isolated.

An effector cell population suitable for use in the present invention can be autogeneic or allogeneic, preferably autogeneic. When effector cells are allogeneic, preferably the cells are depleted of alloreactive cells before use. This can be accomplished by any known means, including, for example, mixing the allogeneic effector cells and a recipient cell population and incubating them for a suitable time, then depleting $CD69^{+}$ cells, or inactivating alloreactive cells, or inducing anergy in the alloreactive cell population.

The effector cell population can comprise unseparated cells, i.e., a mixed population, for example, a PBMC population, whole blood, and the like. The effector cell population can be manipulated by positive selection based on expression of cell surface markers, negative selection based on expression of cell surface markers, stimulation with one or more antigens *in vitro* or *in vivo*, treatment with one or more biological modifiers *in vitro* or *in vivo*, subtractive stimulation with one or more antigens or biological modifiers, or a combination of any or all of these.

Effector cells can be obtained from a variety of sources, including but not limited to, PBMC, whole blood or fractions thereof containing mixed populations, spleen cells, bone marrow cells, tumor infiltrating lymphocytes, cells obtained by leukapheresis, biopsy tissue, lymph nodes, e.g., lymph nodes draining from a tumor.

Suitable donors include an immunized donor, a non-immunized (naïve) donor, treated or untreated donors. A "treated" donor is one that has been exposed to one or more biological modifiers. An "untreated" donor has not been exposed to one or more biological modifiers.

Methods of extracting and culturing effector cells are well known. For example, effector cells can be obtained by leukapheresis, mechanical apheresis using a continuous flow cell separator. For example, lymphocytes and monocytes can be isolated from the buffy coat by any known method, including, but not limited to, separation over Ficoll-Hypaque™ gradient, separation over a Percoll gradient, or elutriation. The concentration of Ficoll-Hypaque™ can be adjusted to obtain the desired population, for example, a population enriched in T cells. Other methods based on cell-specific affinity columns are known and can be used. These include, for example, fluorescence-activated cell sorting (FACS), cell adhesion, magnetic bead separation, and the like. Affinity-based methods can utilize antibodies, or portions thereof, which are specific for cell-surface markers and which are available from a variety of commercial sources, including, the American Type Culture Collection (Rockville, MD). Affinity-based methods can alternatively utilize ligands or ligand analogs, of cell surface receptors.

The effector cell population can be subjected to one or more separation protocols based on the expression of cell surface markers. For example, the cells can be subjected to positive selection on the basis of expression of one or more cell surface polypeptides, including, but not limited to, "cluster of differentiation" cell surface markers such as CD2, CD3, CD4, CD8, TCR, CD45, CD45RO, CD45RA, CD11b, CD26, CD27, CD28, CD29, CD30, CD31, CD40L; other markers associated with lymphocyte activation, such as the lymphocyte activation gene 3 product (LAG3), signaling lymphocyte activation molecule (SLAM), T1/ST2; chemokine receptors such as CCR3, CCR4, CXCR3, CCR5; homing receptors such as CD62L, CD44, CLA, CD146, $\alpha 4\beta 7$, $\alpha E\beta 7$; activation markers such as CD25, CD69 and

OX40; and lipoglycans presented by CD1. The effector cell population can be subjected to negative selection for depletion of non-T cells and/or particular T cell subsets. Negative selection can be performed on the basis of cell surface expression of a variety of molecules, including, but not limited to, B cell markers such as CD19, and CD20; monocyte marker CD14; the NK cell marker CD56.

The effector cell population can be manipulated by exposure, *in vivo* or *in vitro*, to one or more antigens. Antigens include, but are not limited to, peptides; proteins; glycoproteins; lipids; glycolipids; cells; cell extracts; tissue extracts; whole microorganisms such as protozoans, bacteria, and viruses. Antigens can be unmodified, i.e., used in their native state. Alternatively, an antigen can be modified by any known means, including, but not limited to, heating, for example to denature a protein or to inactivate a pathogen; chemical modification to denature a protein, or to cross-link two antigen molecules; glycosylation; chemical modification with moieties including, but not limited to polyethylene glycol; and enzymatic digestion. If more than one antigen is used, the exposure can be simultaneous or sequential.

The effector cells can be cultured in the presence of at least one antigen associated with a condition to be treated. The antigen can be a single antigen with multiple antigenic determinants or can be a mixture of antigens. The antigen can be an autoantigen or a foreign antigen, depending on the condition to be treated. Autoantigens include antigens associated with autoimmune diseases and those associated with cancer cells. The antigen can be a protein, cells, a tissue or a target organ. If the antigen is an autoantigen, the autoantigen can be part of an organ, for example the brain or the thyroid gland and need not be purified therefrom. Purified autoantigens or mixtures of purified autoantigens can also be used.

Co-culturing of peripheral blood leukocytes (PBL) or tumor infiltrating lymphocytes (TIL) with autologous tumor cells is generally accompanied by cytokine stimulation. Sporn et al.(1993) *Cancer Immunol. Immunother.* 37:175-180 ; and Peyret et al. (1991) *Chirurgie* 117:700-709.

An effector cell population can be manipulated by exposure, *in vivo* or *in vitro*, to one or more biological modifiers. Suitable biological modifiers include, but are not limited to, cytokines such as IL-2, IL-4, IL-10, TNF-α, IL-12, IFN-γ; non-specific modifiers such as phytohemagglutinin (PHA), phorbol esters such as phorbol myristate acetate (PMA), concanavalin-A, and ionomycin; antibodies specific for cell surface markers, such as anti-CD2, anti-CD3, anti-IL-2 receptor, anti-CD28; chemokines, including, for example, lymphotactin. The biological modifiers can be native factors obtained from natural sources, factors produced by recombinant DNA technology, chemically synthesized polypeptides or other molecules, or any derivative thereof having the functional activity of the native factor. If more than one biological modifier is used, the exposure can be simultaneous or sequential.

The present invention provides compositions comprising T cells enriched in antigen-specific cells, enriched according to the methods of the invention. By "enriched" is meant that a cell population is at least about 50-fold, more preferably at least about 500-fold, and even more preferably at least about 5000-fold or more enriched from an original mixed cell population comprising the desired cell population. The proportion of the enriched cell population which comprises the desired antigen-specific cells can vary substantially, from less than 10% up to 100% antigen-specific cells. The percentage which are antigen-specific can be readily determined, for example, by a ^{3}H-thymidine uptake assay in which the T cell population is challenged by an antigen-presenting matrix presenting the desired antigen(s).

Cell labeling

The methods herein are based on labeling the cells with a product secreted by the cells, where the product is secreted in response to antigen stimulation. To achieve labeling, the cell surface of a cell population is modified such that a moiety that binds specifically to a product, the "specific binding partner" is attached to the cell surface either directly or through an anchoring means (an "anchor moiety"), optionally

through a linker to form a capture moiety. The cell population can contain numerous types of cells and generally made up of a mixed population. Preferably the cell population is hematopoietic, more preferably the cell population is effector cells, most preferably, the cell population is T cells or a subset thereof. Subsets can be isolated by virtue of cell surface markers, for instance, CD45 for lymphocytes, CD8 for cytotoxic cells, etc.

Products secreted in response to antigen stimulation are known in the art and include, but are not limited to, cytokines, such as IL-2, IL-4, IL-10, TNF-α, TGF-β and IFN-γ.

Specific binding partners include any moiety for which there is a relatively high affinity and specificity between product and binding partner, and in which the dissociation of the product:partner complex is relatively slow so that the product:partner complex is detected during the cell separation technique. Specific binding partners include, but are not limited to, substrates or substrate analogs to which a product will bind, peptides, polysaccharides, steroids, biotin, digitoxin, digitonin and derivatives thereof. In a preferred embodiment the specific binding partner is an antibody or antigen-binding fragment or derivative thereof. The term "antigen-binding fragment" includes any peptide that binds specifically to the product. Typically, these fragments include such immunoglobulin fragments as Fab, $F(ab')_2$, Fab', scFv (both monomer and polymeric forms) and isolated H and L chains. An antigen-binding fragment retains the specificity of the intact immunoglobulin, although avidity and/or affinity can be altered.

In the practice of the invention the capture moiety can be attached to a cell membrane (or cell wall) by a variety of methods. Suitable methods include, but are not limited to, direct chemical coupling to amino groups of the protein components, coupling to thiols (formed after reduction of disulfide bridges) of the protein components, indirect coupling through antibodies (including pairs of antibodies) or

lectins, anchoring in the lipid bilayer by means of a hydrophobic anchor, and binding to the negatively charged cell surface by polycations.

In other embodiments of the invention, the capture moiety is introduced using two or more steps, e.g., by labeling the cells with at least one anchor moiety which allows the coupling of the capture moiety to the anchor moiety either directly, for instance by a biotin/avidin complex or indirectly, through a suitable linking moiety or moieties.

Suitable anchor moieties include lipophilic molecules such as fatty acids. Alternatively, antibodies or other specific binding agents to cell surface markers such as the MHC antigens or glycoproteins, can also be used.

The "capture moiety" can be coupled to the anchor moiety through a linking agent, and can also include a linker which multiplies the number of capture moieties available and thus the potential for capture of product, such as branched polymers, including, for example, modified dextran molecules, polyethylene glycol, polypropylene glycol, polyvinyl alcohol, and polyvinylpyrrolidone.

Methods for direct chemical coupling of antibodies to the cell surface are known in the art, and include, for example, coupling using glutaraldehyde or maleimide activated antibodies. Methods for chemical coupling using multiple step procedures include, but are not limited to, biotinylation, coupling of trinitrophenol (TNP) or digoxigenin using for example succinimide esters of these compounds. Biotinylation can be accomplished by, for example, the use of D-biotinyl-N-hydroxysuccinimide. Succinimide groups react effectively with amino groups at pH values above 7, and preferentially between about pH 8.0 and about pH 8.5. Biotinylation can be accomplished by, for example, treating the cells with dithiothreitol followed by the addition of biotin maleimide.

Coupling to the cells can also be accomplished using antibodies against cell surface antigens ("markers"). Antibodies directed to surface antigens generally require in the range of 0.1 to 1 μg of antibody per 10^7 cells. However, this

requirement will vary widely in response to the affinity of the antibody to the product and will need to be determined empirically. Such a determination is well within the skill of one in the art. Thus, the appropriate amount of antibody must be determined empirically and is within the skill of one in the art. This allows coupling to specific cells on cell type specific marker expression. For instance, classes of cells such as T cells or subsets thereof can be specifically labeled. As a capture moiety, a bispecific antibody can be used which has an antigen recognition site for the cell or an anchor moiety placed thereon, and the product.

A capture moiety, particularly capture antibodies should be selected based on the amount of secreted product. For example, for cells which secrete only a few molecules, a high affinity antibody will catch most of the secreted molecules. Alternatively, in the case where the cell secretes many molecules during the incubation time, a lower affinity antibody can be preferred to prevent too early saturation of the catching matrix. Determination of suitable affinities for the level of proteins secreted are determined empirically and are within the skill of one in the art.

Cells carrying large amounts of N-acetylneuraminic acid on their surface as a constituent of their lipopolysaccharides bear a negative charge at physiological pH values. Coupling of capture moieties can be via charge interactions. For example, moieties bearing polycations bind to negatively charged cells. Polycations are known in the art and include, for example, polylysine and chitosan. Chitosan is a polymer consisting of D-glucosamine groups linked together by α-(1-4) glucoside bonds.

Another method of coupling binding partners (which can comprise one or more capture moieties) to the cells is via coupling to the cell surface polysaccharides. Substances which bind to polysaccharides are known in the art, and include, for example, lectins, including concanavalin A, solanum tuberosum, aleuria aurantia, datura stramonium, galanthus nivalis, helix pomatia, lens culinaris and other known lectins supplied by, a number of companies, including for example, Sigma Chemical Company and Aldrich Chemical Company.

In some embodiments of the invention, the product binding partner is coupled to the cell by hydrophobic anchoring to the cell membrane. Suitable hydrophobic groups that will interact with the lipid bilayer of the membrane are known in the art, and include, but are not limited to, fatty acids and non-ionic detergents (including, e.g., Tween-80). A drawback to attachment of the capture moiety to the cell via the insertion of a hydrophobic anchor is that the rate of integration of the hydrophobic moiety into the cell is low. Thus, high concentrations of the moiety with the hydrophobic anchor often are required. This latter situation is often uneconomical when the capture moiety is a relatively limited or expensive substance, for example, an antibody.

The low yield of hydrophobic molecules that embed themselves in the membrane is relevant only when these molecules are available in relatively limited quantities. This problem can be overcome by using a bridging system that includes an anchoring partner and a partner that contains the capture moiety, wherein one of the partners is of higher availability, and wherein the two parts of the bridging system have a high degree of specificity and affinity for each other. For example, in one embodiment avidin or streptavidin is attached to the cell surface via a hydrophobic anchor, while the partner with the product capture moiety are biotinylated anti-product antibodies. In another embodiment, the cell surface is labeled with digoxigenin followed by conjugates of anti-digoxigenin antibody fragments and anti-product antibodies. This approach can be used with other pairs of molecules able to form a link, including, for example, hapten with antihapten antibodies, NTA with polyhistidine residues, or lectins with polysaccharides. A preferred embodiment is one which allows "amplification" of the system by increasing the number of capture moieties per anchor moiety.

In one illustrative embodiment, a branched dextran is bound to palmitic acid, thus providing a multiplicity of available binding sites. The dextran is in turn

coupled to biotin and treated with avidin-conjugated antibody specific for the product.

It is of course contemplated within the embodiments of the invention that bridging systems can be used between the anchor moiety and the capture moiety when the anchor moiety is coupled in any fashion to the cell surface. Thus, for example, an avidin (or streptavidin) biotin linker moiety can link an antibody anchor moiety with a capture moiety. Bispecific antibody systems can also act as linker moieties.

In order to analyze and, if desired, to select cells that have the capability of secreting the product, cells modified as above to contain the capture moiety are incubated under conditions that allow the production and secretion of the product in a sufficient amount to allow binding to and detection of the cells that contain the captured product. These conditions are known to those of skill in the art and include, inter alia, appropriate temperature, pH, and concentrations of salts, growth factors and substrates in the incubation medium, as well as the appropriate concentrations of gas in the gaseous phase. When it is desirable to distinguish between high and low producer cells, the time of incubation is such that product secretion by the cells is still in a linear phase. The appropriate conditions can be determined empirically and such a determination is within the skill of one in the art.

Additionally, cell secretion can be modified, that is, upregulated, induced, or reduced using a biological modifier. The biological modifiers can be added at any time but are preferably added to the incubation medium. Alternatively, the cells can be pretreated with these agents or cells prior to the incubation step. Suitable biological modifiers include, but are not limited to, molecules and other cells. Suitable molecules include, but are not limited to, drugs, cytokines, small molecules, hormones, combinations of interleukins, lectins and other stimulating agents, e.g., PMA, LPS, bispecific antibodies and other agents that modify cellular functions or protein expression.

Suitable cells include, but are not limited to, direct cell to cell interactions such as between a tumor and T cell and indirect cell to cell interactions such as those induced by the proximity of other cells which secrete a biological modifier. Suitable cells include, but are not limited to, blood cells, peripheral bone marrow cells and various cell lines.

The incubation conditions are also such that product is essentially not captured or is captured to a much lesser extent by another cell, so as to distinguish non-producing cells from product producing cells, or high producers from low producers. Generally the incubation time is between five minutes and ten hours, and is more usually between one and five hours. The incubation medium can optionally include a substance that slows diffusion of the product from the producer cell. Substances which inhibit product diffusion in liquid media and that are non-toxic to cells are known in the art and include a variety of substances that partially or completely gel, including, for example, alginate, low melting agarose and gelatin. By varying the viscosity or permeability of the medium, the local capture by a producing cell of differently sized products can be modulated. The molecular weight size exclusion of the medium can be adjusted to optimize the reaction. The optimal composition of the medium can be empirically determined and is influenced by the cell concentration, the level of secretion and molecular weight of the product and the affinity of the capture moieties for the product. Such determinations are within the skill of one in the art.

Preferably, the gels are solubilized after the incubation to allow the isolation of the cells or groups of cells from the media by cell sorting techniques. Thus, for example, the gels can be linked by disulfide bonds that can be dissociated by sulfhydryl reducing agents such as β-mercaptoethanol or dithiothreitol, or the gels can contain ion cross-linkings, including for example, calcium ions, that are solubilized by the addition of a chelating agent such as EDTA.

At the end of the secretion phase the cells are usually chilled to prevent further secretion, and the gel matrix (if any) is solubilized. This order can, of course, be reversed. As capping can take place after the capture moiety is added due to cross linking, an incubation step to decrease capping can be added at this point. The cells can be incubated for instance in cytochalasin A or B or any other suitable substance that prevents capping. The cells containing the trapped product are then labeled with a label moiety. Labeling can be accomplished by any method known to those of skill in the art. For example, anti-product antibodies can be used to directly or indirectly label the cells containing the product. The labels used are those which are suitable for use in systems in which cells are to be analyzed or sorted based upon the attachment of the label moiety to the product.

In other embodiments, capture moieties that do not contain captured product can be detected. This allows, for example, the isolation of cells that secrete high amounts by employing a negative separation method, i.e., detection of cells not highly saturated with product. The cells can be labeled with other labeling substances recognizing, e.g., cell surface markers, cell type, cellular parameters such as DNA content, cell status, or number of capture moieties.

The enumeration of actual capture moieties can be important to compensate for varying amounts of these molecules due to, for example, different conjugation potentials of the cells. It can be especially important for the isolation of rare cells to exclude cells with decreased or increased capability for binding the product capture system, including the anchor and capture moieties. Alternatively, the reactions can proceed simultaneously in a "one-step reaction."

Cell analysis and cell sorting

Analysis of the cell population and cell sorting based upon the presence of the label can be accomplished by a number of techniques known in the art. Cells can be analyzed or sorted by, for example, flow cytometry or FACS. These techniques allow the analysis and sorting according to one or more parameters of the cells.

Usually one or multiple secretion parameters can be analyzed simultaneously in combination with other measurable parameters of the cell, including, but not limited to, cell type, cell surface markers, DNA content, etc. The data can be analyzed and cells sorted using any formula or combination of the measured parameters. Cell sorting and cell analysis methods are known in the art and are described in, for example, *The Handbook of Experimental Immunology*, Volumes 1 to 4, (D.N. Weir, editor); *Flow Cytometry Cell Sorting* (A. Radbruch, editor, Springer Verlag, 1992); and *Cell Separation Methods and Applications* (D. Recktenwald and A. Radbruch, eds., 1997) Marcel Dekker, Inc. N.Y. Cells can also be analyzed using microscopy techniques including, for example, laser scanning microscopy, fluorescence microscopy; techniques such as these can also be used in combination with image analysis systems. Other methods for cell sorting include, for example, panning and separation using affinity techniques, including those techniques using solid supports such as plates, beads and columns.

Some methods for cell sorting utilize magnetic separations, and some of these methods utilize magnetic beads. Different magnetic beads are available from a number of sources, including for example, Dynal (Norway), Advanced Magnetics (Cambridge, MA, U.S.A.), Immuncon (Philadelphia, U.S.A.), Immunotec (Marseilles, France), and Miltenyi Biotec GmbH (Germany).

Preferred magnetic labeling methods include colloidal superparamagnetic particles in a size range of 5 to 200 nm, preferably in a size of 10 to 100 nm. These magnetic particles allow a quantitative magnetic labeling of cells, thus the amount of coupled magnetic label is proportional to the amount of bound product, and the magnetic separation methods are sensitive to different amounts of product secretion. Colloidal particles with various specificities are known in the art, and are available, for example, through Miltenyi Biotec GmbH. The use of immunospecific fluorescent or magnetic liposomes can also be used for quantitative labeling of captured product. In these cases, the liposomes contain magnetic material and/or fluorescent dyes

conjugated with antibody on their surfaces, and magnetic separation is used to allow optimal separation between nonproducing, low producing, and high producing cells.

The magnetic separation can be accomplished with high efficiency by combining a second force to the attractive magnetic force, causing a separation based upon the different strengths of the two opposed forces. Typical opposed forces are, for example, forces induced by magnetic fluids mixed in the separation medium in the magnetic separation chamber, gravity, and viscous forces induced by flow speed of medium relative to the cell. Any magnetic separation method, preferably magnetic separation methods allowing quantitative separation will be used. It is also contemplated that different separation methods can be combined, for example, magnetic cell sorting can be combined with FACS, to increase the separation quality or to allow sorting by multiple parameters.

Preferred techniques include high gradient magnetic separation (HGMS), a procedure for selectively retaining magnetic materials in a chamber or column disposed in a magnetic field. In one application of this technique the product is labeled by attaching it to a magnetic particle. The attachment is generally through association of the product with a label moiety which is conjugated to a coating on the magnetic particle which provides a functional group for the conjugation. The captured product thus coupled to a magnetic "label", is suspended in a fluid which is then applied to the chamber. In the presence of a magnetic gradient supplied across the chamber, the magnetically labeled target cell is retained in the chamber; if the chamber contains a matrix, it becomes associated with the matrix. Cells which do not have or have only a low amount of magnetic labels pass through the chamber.

The retained cells can then be eluted by changing the strength of, or by eliminating, the magnetic field or by introducing a magnetic fluid. The selectivity for a captured product is supplied by the label moiety conjugated either directly or indirectly to the magnetic particle or by using a primary antibody and a magnetic particle recognizing the primary antibody. The chamber across which the magnetic

field is applied is often provided with a matrix of a material of suitable magnetic susceptibility to induce a high magnetic field gradient locally in the camber in volumes close to the surface of the matrix. This permits the retention of fairly weakly magnetized particles. Publications describing a variety of HGMS systems are known in the art, and include, for example, U.S. Patent No. 4,452,773, U.S. Patent No. 4,230,685, PCT application WO85/04330, U.S. Patent No. 4,770,183, and PCT/EP89/01602; systems are also described in U.S. Patent Nos. 5,411,863; 5,543,289; 5,385,707; and 5,693,539, which are commonly owned and hereby incorporated herein by reference.

In addition, in other embodiments the processes include labeling the cells that contain the product captured by the capture moiety, if any. Other embodiments can also include analyzing the cell population to detect labeled cells, if any, and if desired, sorting the labeled cells, if any.

Diagnostic methods for detecting antigen-specific T cells

The present invention further provides diagnostic methods for detecting antigen-specific T cells. These include methods for analyzing a population of cells enriched for T cells to identify or enumerate antigen-specific T cells, as well as methods of determining a distribution of antigen-specific T cells that secrete a product in response to antigen stimulation.

Methods for analyzing a population of cells enriched in T cells to identify or enumerate antigen-specific T cells that secrete and release an amount of product relative to other cells in the population, wherein the product is secreted and released in response to antigen stimulation, comprise the steps of labeling the cells by the methods of the present invention; labeling the cells with at least one additional label that does not label the captured product; and detecting the amount of product label relative to the additional label. Such methods are useful, for example, in determining the proportion of a cell population that is specific for a given antigen. The method can be used to provide information regarding the immune status of an individual,

including assessing an immune response to allergens, a tumor or virus, or evaluating the proportion of cells in an individual that are self reactive so as to detect or monitor autoimmune diseases.

Method of treatment using enriched antigen-specific T cells

The present invention provides methods of treatment of a disease or condition related to a population of antigen-specific T cells, using the enriched T cells of the invention.

Treatment methods include those in which an antigen-specific T cell population is identified, enriched, and introduced into an individual; those in which a population of antigen-specific T cells is identified, enriched and expanded *in vitro* before introduction into an individual; those in which a population of antigen-specific T cells is identified and eliminated from a population of cells to be introduced into an individual; ex vivo genetic modification prior to administration; and selection of antigen-specific T cells selected according to cytokine expression. Examples of antigen-specific T cells selected according to cytokine expression include, but are not limited to, IFN-γ or TNF-α secreting $CD8^+$ T cells (cytotoxic) for treatment of cancer, viral (e.g. CMV, EBV) and bacterial (e.g. listeria, mycobacteria) infections; IFN-γ secreting $CD4^+$ T cells for the same indications and also for suppression and/or counter-regulation of allergy or vaccination against allergy, suppression of TH2-associated autoimmune diseases or vaccination against these autoimmune diseases; IL-10 or TGF-beta secreting $CD4^+$ T cells, for suppression TH1, but also TH2-associated autoimmune diseases or vaccination against these autoimmune diseases (tolerance induction); IL-4 secreting $CD4^+$ T cells for suppression of TH1-associated autoimmune diseases or vaccination against these autoimmune diseases; and IL-4 or IL-5 secreting $CD4^+$ T cells for treatment of helminth infections.

T cell populations enriched according to the methods of the present invention can be used to treat a variety of disorders. Included among these are cancer. T cells specific for a tumor antigen can be obtained using the methods of the present

invention. Tumor cells can be obtained from an individual, and these can be co-cultured *in vitro* with T cells obtained from the same individual. After co-culturing the cells for a suitable time, tumor-specific T cells can be enriched according the methods of the present invention. This enriched population can then be re-introduced into the patient. Methods for anti-tumor immunotherapy using autologous T cells are known in the art. See, for example, WO 97/05239.

Alternatively, cells used in anti-tumor immunotherapy treatments can be allogeneic. Various modes of treatment of cancer with allogeneic T cells have been described in the art and can be used in the methods of the present invention. See, for example, PCT Publication No. WO 96/37208. Optionally, allogeneic T cells can be activated prior to introduction into an individual. Activation can be effected through contact with a biological modifier, an antibody directed to a cell surface marker, or a ligand or analog thereof for a cell surface receptor.

Another use of enriched T cell populations of the present invention is in immunomodulation, for example, in the treatment of autoimmune disorders, inflammatory disorders, allergies and hypersensitivities such as delayed-type hypersensitivity and contact hypersensitivity. T cells which are capable of destroying or suppressing the activity of autoreactive cells can be enriched *in vitro*, optionally expanded *in vitro*, then re-introduced into a patient. In the treatment of allergic responses, the ratio of TH1 to TH2 cells can be altered, or, cells reactive toward allergen-specific cells can be enriched and introduced into an individual.

Inducing T cell anergy can also be used to treat, ameliorate or prevent allograft rejection thus improving the results of organ transplantation and increasing the range of histotypes to which a patient can be made histocompatible.

Compositions comprising enriched T cell populations can further be used as vaccines, to prevent or substantially reduce the probability of the occurrence of a disease state such as a viral infection, autoimmune disorder, allergic response, cancer,

or other disorder, or will reduce the severity or duration of the disease if subsequently infected or afflicted with the disease.

The compositions of cells can be administered by any known route, including, but not limited to, intravenously, parenterally, or locally. In the treatment methods of the present invention, enriched T cells are administered to an individual. The total number of cells, the number of doses, and the number of cells per dose will depend upon the condition being treated. Generally, about 10^6 to 10^{11} cells are administered in a volume ranging from about 5 ml to 1 liter. The cells can be administered in a single dose or in several doses over selected time intervals. Of the cells being administered, preferably at least about 10%, more preferably at least about 20%, more preferably at least about 50%, are antigen-specific T cells which secrete a product.

Kits

It is contemplated that the reagents used in the detection of secretor cells of desired products can be packaged in the form of kits for convenience. The kits would contain, for example, optionally one or more materials for use in preparing gelatinous cell culture medium, the medium to be used for cell incubation for the production of the desired secreted product; a product capture system comprised of anchor and capture moieties; a label moiety; and instructions for use of the reagents. All the reagents would be packaged in appropriate containers.

The kit can also be formulated to include the following. In this case all the reagents are preferably placed in a single vial to which the cells are added. At least one antibody which is bispecific for a particular cell surface structure or anchor moiety and the product. At least one label moiety and, optionally, biological modifiers.

Optionally, the kit can include physiologically acceptable buffer. Such buffers are known in the art and include, but are not limited to, PBS with and without BSA, isotonic saline, cell culture media and any special medium required by the

particular cell type. Buffers can be used that reduce cross-labeling and increase the local product concentration around the cells. Buffers can include agents for increasing viscosity or decreasing permeability. Suitable agents are described herein. The viscosity of the medium can be reduced before analysis by any method known in the art including, but not limited to, dissolution in a physiologically acceptable buffer, dissolving heat, EDTA, and enzymes. In the absence of added medium, cells already suspended in a medium can be directly added to the vial. Suitable cell suspensions include but are not limited to cell lines and biological samples. Biological samples include, but are not limited to, blood, urine and plasma.

Additional structures can be added for catching unbound product to reduce cell cross-contamination thereby reducing the diffusion of products away from the producing cells. These include, but are not limited to, anti-product antibody immobilized to gel elements, beads, magnetic beads, and polymers.

Biological modifiers can also be added to the buffer or medium to induce specific secretion.

Additional label moieties such as antibodies (magnetically or fluorescently labeled) can also be present, including, but not limited to anti-cell surface marker antibodies to identify cell types, propidium iodide to label dead cells, and magnetic beads to label certain cell types.

In this embodiment, all materials can be placed in a single container such as a vial and the cell sample added. The contents are incubated to allow secretion of a product and subsequent capture of the product and binding of the label moiety to the product. The cells which have secreted and bound product can then be separated and/or analyzed based on the presence, absence or amount of the captured product. Separation can be done by any of the methods known in the art, including, but not limited to, simple dilution, erythrocyte lysis, centrifugation-washing step, magnetic separation, FACS and Ficoll separation. The analysis of the cells can be performed

by a variety of methods, including, but not limited to, FACS, image analysis, cytological labeling, and immunoassay.

The following examples are provided solely for the purposes of illustration and not to limit the scope of the invention. In light of the present disclosure, numerous embodiments within the scope of the claims will be apparent to those of ordinary skill in the art.

Example 1

Peripheral blood mononuclear cells (PBMC) were cultured in complete RPMI 1640 (Gibco BRL, Grand Island, NY) containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.3 mg/ml glutamine, 10 mM 2-mercaptoethanol and 10% human serum type AB (Sigma, St. Louis, MO) at a cell concentration of 2×10^6 cells/ml. Peptide Ml 58-66 from Influenza virus matrix protein (GILGFVFTL; Neosystem, Strasbourg, France) was added to a final concentration of 1μM. Control cells were cultured without peptide.

Cells were incubated at 37°C in an atmosphere containing 7.5% CO_2. After 5 hours and 30 minutes, cells were harvested by centrifugation. Cells were incubated at a cell concentration of $5x10^7$ cells/ml in complete RPMI 1640 with anti human interferon gamma (IFN-γ) monoclonal antibody (mAb) 4SB3 conjugated to anti-human CD45 mAb 5Bl (30 μg/ml) at 8°C for 7 min. The cells were then diluted to 2 x 10^6 cells/ml with complete RPMI 1640 containing 10% FCS and incubated for 45 minutes at 37°C. Then cells were pelleted and incubated with phycoerythrin (PE)-conjugated anti human interferon gamma (IFN-γ) mAb NIB42 (4 μg/ml) and FITC-labeled anti-CD8 mAb in PBS/BSA/EDTA solution 0.05% BSA and 2mM EDTA, for 10 minutes at 4°C. Cells were then washed in PBS/BSA/EDTA and labeled with mouse anti-PE mAb 80-5 conjugated to MicroBeads (Miltenyi Biotec) in PBS/BSA/EDTA for 15 minutes at 8°C. Cells were washed and resuspended in 500μl PBS/BSA/EDTA.

IFN-γ-secreting cells were enriched with the magnetic cell separation system MACS. Magnetically labeled cell suspension was pipetted onto a MiniMACS separation column in a MiniMACS separation unit, the cell suspension was allowed to pass through and the column was washed with 3 x 500µl buffer. The effluent was collected as negative fraction (N1). The column was removed from the separator, and placed on a suitable tube. 1 ml buffer was pipetted on top of column and magnetically labeled cells were flushed out using a plunger and applied to a second round of MiniMACS separation.

The original cells (*i.e.*, before MACS separation), negative cell fractions (of first as well as second MACS separation, designated N1 and N2, respectively) and positive cell fraction (P2) of second MACS separation were analyzed by flow cytometry. FACScan and CELLQuest research software (Becton Dickinson, Mountain View, CA) were used for flow cytometric analysis. Dead cells and cell debris were excluded according to scatter properties and staining with propidium iodide (PI; 0.3 µg/ml).

The results are shown in Figures 1A-P. While dot plots A-H show analysis of control cells cultured without peptide, plots I-P show analysis of peptide stimulated cells. Dot plots show the scatter properties of the starting cell population (A and I) and the enriched cell populations (C and K); and PI versus PE fluorescence of the starting cell population (B and J) and enriched cell population (D and L).

Dot plots E-H and M-P show anti-CD8-FITC versus anti-IFN-γ-PE staining of gated cells in original (E and M), first negative (F and N), second negative (G and O) and in the final positive cell fraction (H and P).

While in the control cell population, $CD8^+$ IFN-γ^+ cells were enriched up to 11 % among live cells (Figure 1H), in the peptide stimulated cell population, $CD8^+$ IFN-γ^+ cells were enriched up to 40% (Figure 1P). From a starting population of 3.5 x 10^7 control cells, about 600 $CD8^+$ IFN-γ^+ cells were isolated, compared to 4100

CD8+ IFN-γ^+ cells isolated from a starting population of 3.5 x 10^7 peptide-stimulated cells.

CD8- cells brightly stained with PE-labeled anti-IFN-γ were CD19$^+$ B cells, most likely B cells specific for a sorting reagent, probably PE. These cells were enriched to the same extent from control cells compared to peptide stimulated cells.

Also the CD8$^-$ cells dimly stained with PE-labeled anti-IFN-γ (like the CD8$^+$ IFN-γ^+ cells) were enriched to the same extent from control cells compared to peptide stimulated cells. Such cells partially stain for CD4 and CD56, and therefore are most likely T helper cells or NK cells secreting IFN-γ.

Thus there is a basal level of IFN-γ secretion by (CD4+) T helper cells, (CD8$^+$) cytotoxic T cells and (CD56$^+$) NK cells without intentional antigen-specific stimulation *in vitro,* which reflects most likely the IFN-γ secretion induced already *in vivo* in ongoing immune responses at the time of blood sampling.

However, IFN-γ^+-secreting CD8$^+$ cells induced by stimulation with the HLA class I-restricted influenza peptide Ml 58-66 were significantly enriched above this background level; therefore, most of the CD8+ IFN-γ^+ cells enriched from peptide stimulated cells are peptide-specific T cells. Specificity of enriched cells was further confirmed by staining for the presence of Vβ17 TCR, which is a conserved T cell receptor (TCR) segment in Ml 58-66 specific cytotoxic T cells. Lehner et al. (1995) *J. Exp. Med.* 181:79-91; and Lalvani et al. (1997) *J. Exp. Med.* 186:859-865. Among IFN-γ^+ cells isolated from peptide stimulated cells, but not among IFN-γ^+ cells isolated from control cells, most express Vβ17$^+$ TCRs.

Example 2

Peripheral blood mononuclear cells (PBMC) were cultured in complete RPMI 1640 (Gibco BRL, Grand Island, NY) containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.3 mg/ml glutamine, 10 mM 2-ME and 10% human serum type AB (Sigma, St. Louis, MO) at 2 x 10^6 cells/ml. Peptide M1 58-66 from Influenza virus

matrix protein (GILGFVFTL; Neosystem, Strasbourg, France) was added to a final concentration of 1 μM. Control cells were cultured without peptide.

After 5 hours and 30 minutes cells were harvested by centrifugation. Cells were incubated at $5x10^7$ cells/ml in complete RPMI 1640 with anti-human IFN-γ mAb 4SB3 conjugated to anti-human CD45 mAb 5B1 (30 μg/ml) at 8°C for 7 minutes. The cells were then diluted to $2x10^6$ cells/ml with complete RPMI 1640 containing 10% FCS and incubated for 45 minutes at 37°C. Then cells were spun down and incubated with phycoerythrin (PE)-conjugated anti-human -IFN-γ mAb NIB42 (4 μg/ml) and FITC-labeled anti-CD8 in PBS/BSA/EDTA, for 10 minutes at 4°C. Cells were then washed in PBS/BSA/EDTA and labeled with mouse anti-PE mAb 80-5 conjugated MicroBeads (Miltenyi Biotec) in PBS/BSA/EDTA for 15 minutes at 8°C. Cells were washed and resuspended in 500 μl PBS/BSA/EDTA.

IFN-γ-secreting cells were enriched with the magnetic cell separation system MACS. Magnetically labeled cell suspension was pipetted on top of a MiniMACS separation column in a MiniMACS separation unit, cell suspension was allowed to pass through and column was washed with 3 x 500 μl buffer. Effluent was collected as negative fraction. The column was removed from separator, and placed on a suitable tube. 1 ml buffer was pipetted on top of column and magnetically labeled cells were flushed out using a plunger and applied to a second round of MiniMACS separation.

Original cells (i.e., before MACS separation), negative cell fractions (of first as well as second MACS separation) and positive cell fraction of second MACS separation were analyzed by flow cytometry. FACScan and CELLQuest research software (Becton Dickinson, Mountain View, CA) were used for flow cytometric analysis. Dead cells and cell debris were excluded according to scatter properties and staining with propidium iodide (PI; 0.3 μg/ml) as shown in Example 1. The results are shown in Figure 2.

While dot plots 2A-G show analysis of control cells cultured without peptide, plots 2J-R show analysis of peptide stimulated cells.

Dot plots 2A-D and 2J-M show FITC-labeled anti-CD8 versus PE-labeled anti-IFN-γ staining of gated cells in original (A, J), first negative (B, K), second negative (C, L) and in the final positive cell fraction (D, M).

In the control cells $CD8^+$ IFN-γ+ cells were enriched up to 8.2% among live cells (2D), out of peptide stimulated cells $CD8^+$ IFN-γ^+ cells were enriched up to 41.6% (2M). Out of 6.1 x 10^7 control cells, about 1360 $CD8^+$ IFN-γ^+ cells were isolated compared to 11700 $CD8^+$ IFN-γ^+ cells out of 6.9 x 10^7 peptide stimulated cells.

IFN-γ^+ secreting $CD8^+$ cells induced by stimulation with the HLA class I-restricted influenza peptide Ml 58-66 were significantly enriched above background level, i.e., most of the $CD8^+$ IFN-γ^+ cells enriched from peptide stimulated cells must be peptide-specific T cells. Specificity of enriched cells was further confirmed by staining against Vβ17 TCR, which is a conserved T cell receptor (TCR) segment in Ml 58-66 specific cytotoxic T cells (Lehner 1995; Lalvani 1997). Only among IFN-γ^+ cells isolated from peptide stimulated cells, but not among IFN-γ^+ cells isolated from control cells, most express V$\beta17^+$ TCRs (2F versus 2O).

The following examples show that appropriate antigen-specific stimulation, $CD4^+$ and $CD8^+$ lymphocytes rapidly express cytokines. The technique is demonstrated here for HLA-A0201-restricted influenza matrix protein (FLU) peptide 58-66-specific $CD8^+$ cytotoxic T lymphocytes (CTLs), influenza A virus- and recombinant tetanus toxin C (rTT.C)-fragment-specific T helper type 1 (Th1) cells, and tetanus toxoid (TT) specific T helper type 2 (Th2) cells.

Example 3

Materials and Methods for Examples 4-8

Cells and ex vivo stimulation

Buffy coats were obtained from the Institute for Transfusions medicine, Hospital Merheim, Cologne, Germany and, if necessary, selected on the basis of HLA-type. PBMC were prepared by standard Ficoll-Pacque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, washed twice in PBS and resuspended at a cell concentration of 2 x 10^6 cells per ml in cell culture medium consisting of RPMI 1640 (Life Technologies, Paisley, UK) supplemented with 10% (wt/vol) human AB-serum (Boehringer Ingelheim, Ingelheim, Germany), 1 mM L-alanyl-glutamine (Life Technologies), 100 U/ml penicillin/streptomycin (Life Technologies), 0.05 mM 2-mercaptoethanol (Life Technologies) and 1 mM sodium-pyruvate (Life Technologies). 12.5 ml of the cell suspension were place in 100 x 20 mm tissue culture dishes (Sarstedt, Newton, MA) and FLU 58-66 peptide (Neosystems, Strasbourg, France) was added to a final concentration of 1 μM, purified influenza A virus preparation (Biodesign, Kennebunk, ME) was added to a final concentration of μg/ml, rTT.C (Boehringer Mannheim, Mannheim, Germany) was added to a final concentration of 7 μg/ml and purified TT (Statens Serum Institut, Copenhagen, Denmark) was added to a final concentration of 1 μg/ml. Cells were incubated at 37°C in a humidified 7.5% CO_2 atmosphere for 5-10 h.

Capturing of secreted cytokines by cellular affinity matrices

Ab-Ab conjugates directed against CD45 and either IL-4 or IFN-γ were produced by standard protein coupling techniques. Aslam et al. (1998) *Bioconjugation*, Macmillan Reference Ltd., London. After the *ex vivo* stimulation, cells were harvested using a disposable cell scraper (Costar, Cambridge, MA) and labeled for 7 min at a cell concentration of 10^8 cells per ml in ice-cold medium with 50 μg per ml of the Ab-Ab conjugates. Then, cells were diluted with medium to a

final cell concentration of 2 x 10^6 cells per ml and allowed to secrete for 45 min at 37°C in a humidified 7.5% CO_2 atmosphere.

Magnetic enrichment and detection of cytokine secreting cells

After the cytokine capturing period, cells were harvested again, resuspended at a cell concentration of 10^8 cells per ml in phosphate-buffered saline containing 0.5% (w/v) bovine serum albumin and 5 mM EDTA (buffer) and stained for 10 min at +4°C with 5 µg/ml anti IFN-γ-PE or anti IL-4-PE, respectively. Cells were washed with buffer (300 x g, 10 min), resuspended in 400 µl buffer and magnetically labeled for 15 min at +4°C with 100 µl anti PE Ab-microbeads (Miltenyi Biotec, Bergisch, Gladbach, Germany). After washing, the cells were applied onto a MS+ column and placed in a MiniMACS magnet (Miltenyi Biotech). The column was rinsed with buffer and the retained cells were eluted from the column after removing it from the magnetic field to achieve a higher enrichment rate, the eluted cells from the first column were applied to another MS+ column and the magnetic separation was repeated. Cell samples were analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) using the CellQuest software package.

Magnetic enrichment and detection of cytokine secreting cells

For detection, enumeration and phenotyping of cytokine-secreting cells the following reagents were used: anti IFN-γ-CD45 (anti IFN-γ, clone 4SB3; CD45, clone 5B1, W. Knapp, Vienna, Austria), anti IFN-γ-PE (clone 45-15), anti IL-4-CD45 (anti IL-4, clone 1 A6-10; CD45, clone 5B1, W. Knapp Vienna, Austria), anti IL-4-PE (clone 7A3-3), CD8-Cy5 (clone BM135/80, Behring Diagnostics, Marburg, Germany), CD4-Cy5 (clone M-T321, Behring), CD4-FITC (clone SK3, Becton Dickinson), CD27-FITC (clone M-T271, Pharmingen, San Diego, CA), CD28-FITC (clone CD28.2, Pharmingen) CD57-FITC (clone HNK-1, Becton Dickinson), anti Vβ17.FITC (clone E17.5F3.15.13, Coulter-Immunotech, Marseille, France). Meager et al. (1984) *Interferon Res.* 4:619-625; Alkan et al. (1994) *J. Immunoassay* 15:217-225; and Bird et al. (1991) *Cytokine* 3:562-567.

Cytolytic activity assay

The cytotoxic activity of enriched cytokine-secreting cells was analyzed using a flow cytometry-based assay which has been described previously. Mattis et al. (1997) *J. Immunol. Met.* 204:135-142. Briefly, 1 x 10^6 HLA-A2.1$^+$ T2 cells were labeled with 4 μg per ml of the green fluorescent dye DiO (Molecular Probes, Eugene, OR) in phosphate-buffered saline containing 5 mM EDTA and 3% fetal calf serum for 45 min at 37°C. Cells were washed three times with buffer, resuspended in cell culture medium and loaded with 1 μM Flu 58-66 peptide or Melan A/MART 1 27-35 peptide (Bachem, Heidelberg, Germany) overnight at 37°C in a humidified 7.5% CO_2 atmosphere. Enriched cytokine-secreting cells were expanded for 18 d in tissue culture in the presence of recombinant human IL-2 (Peprotech, London, U.K.). Expanded cytokine-secreting cells and peptide-loaded DiO-labeled HLA-A2.1$^+$ T2 cells were co-cultivated for 16 h at a ratio of 1:1 at 37°C in a humidified 7.5% CO_2 atmosphere. After the culture period, cells were harvested and analyzed by flow cytometry. In order to permit discrimination between live and dead DiO-labeled T2 cells, samples were counterstained with the red fluorescent exclusion dye propidium iodide.

Example 4

The capability to secrete effector cytokines like IFN-γ following short-term antigenic restimulation with synthetic peptide- or native antigen-pulsed APCs is a typical feature of memory/effector CD4$^+$ (Th1-type) and CD8$^+$ T cells. Salmon et al. (1989) *J. Immunol.* 143:907-912; and Hamaan et al. (1997) 186:1407-1418. To isolate low-frequency memory/effector antigen-specific CD4$^+$ and CD8$^+$ T cells directly from peripheral blood based on antigen-induced secretion of IFN-γ and cellular affinity matrix technology, peripheral blood mononuclear cells (PBMC) from HLA-matched adult healthy blood donors were stimulated for 5-6 h with: (a) the HLA-A0201-restricted FLU peptide 58-66, (b) a purified influenza A virus preparation and (c) rTT.C. After the stimulation period, an affinity matrix for IFN-γ was created on the cell surface using antibody (Ab)-Ab conjugates directed against

CD45 and IFN-γ, and the cells were allowed to secrete IFN-γ in culture for 45 min. Then, IFN-γ, relocated to the affinity matrix of the secreting cells, was stained with a phycoerythrin (PE)-conjugated IFN-γ-specific Ab, and PE-labeled cells were enriched by MACS using anti PE Ab microbeads. See, also, Brosterhus et al., 10th International Congress in Immunology, New Delhi, India, 1-6 Nov. 1998, pp. 1469-1473.

Compared with the non-stimulated control samples, a significantly higher proportion of IFN-γ-secreting $CD8^+$ cells were detectable after enrichment in the FLU 58-66 peptide-stimulated sample (Fig. 3A: 38.3% vs. 13.7%), and significantly higher proportions of IFN-γ-secreting $CD4^+$ cells were detectable after enrichment in the samples stimulated with the influenza A virus preparation (Fig. 3B: 35.5% vs. 1.1%) and rTT.C (Fig. 3C: 6.1% vs. 0.3%), respectively. When looking at the absolute numbers of enriched IFN-γ-secreting T cells and their frequencies among total PBMC, differences between the stimulated and non-stimulated samples are even more remarkable: (a) 12,500 IFN-γ-secreting $CD8^+$ T cells were isolated from 5.3×10^7 FLU 58-66 peptide-stimulated PBMC (frequency 1 in 4,200) and 1370 IFN-γ-secreting $CD8^+$ T cells were isolated from 5.1×10^7 non-stimulated PBMC (frequency: 1 in 37,000); (b) 351 IFN-γ-secreting $CD4^+$ T cells were isolated from 5×10^6 influenza A virus-stimulated PBMC (frequency 1 in 14,000) and 4 IFN-γ-secreting $CD4^+$ T cells were isolated from 5.0×10^6 non-stimulated PBMC (frequency 1 in 1,250,000); and (c) 132 IFN-γ-secreting $CD4^+$ T cells were isolated from 1.8×10^7 rTT.C-stimulated PBMC (frequency: 1 in 136,000) and 7 IFN-γ-secreting $CD4^+$ T cells were isolated from 1.9×10^7 non-stimulated PBMC (frequency: ~1 in 2,710,000). Considering these experimental results, it is evident that IFN-γ-secreting T cells present at frequencies of below 10^{-6} can be detected with our technique.

Example 5

Both memory-and effector-type $CD8^+$ T cells are capable of secreting IFN-γ. Hamann et al. (1997). To determine the phenotype of FLU 58-66 peptide-specific $CD8^+$ T cells, enriched IFN-γ-secreting $CD8^+$ T cells from the FLU 58-66 peptide-stimulated sample and the control sample were analyzed by three-color immunofluorescence for the expression of a panel of leukocyte surface markers that allow to distinguish between memory and effector-type $CD8^+$ T cells. Hamann et al. As shown in Figure 2, most FLU 58-66 peptide-specific $CD8^+$ T cells were (1997) $CD27^+$, $CD28^+$ and $CD57^-$, consistent with a memory phenotype, whereas most of the IFN-γ-secreting $CD8^+$ T which became isolated independent of the FLU 58-66 peptide were $CD27^-$, $CD28^-$, $CD57^+$, consistent with an effector phenotype. The latter could have been induced *in vivo* to secrete IFN-γ and thus might reflect ongoing immune responses.

More than 54.8% of the IFN-γ-secreting $CD8^+$ T cells from the FLU 58-66 peptide-stimulated sample expressed the Vβ17 TCR chain, compared with less than 2.2% of the IFN-γ-secreting $CD8^+$ T cells from the control sample (Fig. 4). This confirms previous reports showing a bias of HLA-A0201-restricted FLU peptide 58-66-specific $CD8^+$ T cells towards the use of Vβ17 TCR chain, first in cloned CTLs and later, using fluorescent tetramers of FLU 58-66 peptide-loaded HLA-A2.1 molecules, also in PBMC. Lehner et al. (1995) .*J. Exp. Med.* 181:79-91; and Dunbar et al. (1998).

Example 6

To further confirm the specificity of the enriched IFN-γ-secreting $CD8^+$ T cells from the FLU 58-66 peptide-stimulated PBMC, and to study their cytolytic activity, the cells were expanded for 18 d in tissue culture in the presence of IL-2, and then assayed for CTL activity at an effector: target ratio of 1:1. As shown in Figure 5, significant killing was observed when target cells were loaded with FLU 58-66 peptide, but not when target cells were loaded with a control peptide (Melan A/MART 1 27-35).

Example 7

PBMC from 49 HLA-A2+ individuals were cultured with or without the FLU 58-66 peptide and subjected to the enrichment procedure for IFN-γ-secreting cells as described in Example 3. In 45 cases, on average about 80-fold more IFN-γ-secreting $CD8^+$ T cells were isolated from the FLU 58-66 peptide-stimulated sample as compared to the control sample. Only in three cases, no significant difference was detected between both samples. The median frequency of FLU 58-66 peptide-specific $CD8^+$ T cells among PBMC, as determined by subtracting the frequencies of the control samples from the frequencies of the FLU 58-66 peptide-stimulated samples, was 1 in 30,000 (range between 1 in 600,000 and 1 in 1000). These results are completely consistent with previous reports in which the frequencies of FLU 58-66 peptide-specific $CD8^+$ T cells were determined using enzyme-linked immunospot (ELISPOT) assays for single cell IFN-γ release or tetramers of FLU 58-56 peptide-loaded HLA-A2.1 molecules. Lalvani et al. (1997; and Dunbar et al. (1998).

Example 8

To demonstrate that our approach isolates live antigen-specific Th2-type $CD4^+$ T cells, PBMC were stimulated with purified TT and IL-4-secreting $CD4^+$ T Cells were isolated using an Ab-Ab conjugate directed against CD45 and IL-4. After

10 h of TT stimulation, 150 IL-4-secreting $CD4^+$ T cells could be isolated from 2.2 x 10^7 PBMC with a purity of 6,89% (Fig. 6). This corresponds to a frequency of TT-specific Th2 cells among total $CD4^+$ T cells of 1 in 94,000. The frequency of IL-4-secreting $CD4^+$ T Cells in the control culture without TT was about 10 times lower.

All references cited herein, both supra and infra are hereby incorporated herein. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent to those skilled in the art that certain changes and modifications can be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

Claims

1. A method for obtaining a cell population enriched in antigen-specific T cells, comprising the steps of:

a) obtaining a mixed population of cells comprising T cells;

b) exposing the cell population to at least one antigen under conditions effective to elicit antigen-specific stimulation of at least one T cell and allowing expression of at least one product by the stimulated T cell, wherein the product is secreted in response to antigen stimulation;

c) modifying the surface of the cells to contain a capture moiety specific for the product such that the capture moiety is coupled to the cell surface;

d) culturing said population under conditions wherein said product is secreted, released and specifically bound to the capture moiety, thereby labeling the product-secreting cells; and

e) separating the cells according to the degree to which they are labeled with said product to obtain a population of cells substantially enriched in antigen-specific T cells,

wherein steps (b) and (c) can be performed in any order.

2. A method according to claim 1, further comprising the step of labeling the product prior to separation.

3. The method according to claim 2 wherein the product is labeled with a label moiety.

4. The method according to claim 3 wherein the label moiety is an antibody specific for the product.

5. The method according to claim 3 wherein the label moiety is fluorochromated and the separation is conducted by cell sorting.

6. The method according to claim 3 wherein the label moiety is magnetizable and the separation is conducted in a magnetic field of sufficient strength to magnetize the label moiety.

7. The method according to claim 6 wherein the label moiety comprises colloidal magnetic particles with a typical diameter of about 5 to 200 nm.

8. The method according to claim 1 wherein the capture moiety is an antibody or an antigen-binding fragment thereof.

9. The method according to claim 8 wherein the antibody or antigen binding fragment thereof is bispecific.

10. The method according to claim 1 wherein the coupling is through a lipid anchor attached to the capture moiety optionally through a linking moiety.

11. The method according to claim 1 wherein the coupling is through an antibody or an antigen-binding fragment thereof attached to the capture moiety, optionally through a linker.

12. The method according to claim 1 wherein the coupling is through direct chemical coupling of the capture moiety to components on the cell surface, optionally through a linker.

13. The method according to claim 9 wherein the coupling is through specific binding of the antibody to the cell.

14. A method to label antigen-specific T cells with a product secreted and released by the cells, wherein the product is secreted in response to antigen stimulation, which method comprises:

exposing the cells to at least one antigen under conditions effective to elicit antigen-specific stimulation of at least one T cell; and

modifying the surface of the cells to contain a capture moiety specific for the product; and

culturing the cells under conditions wherein the product is secreted, released and specifically bound to the capture moiety, thereby labeling the product-secreting cells.

15. The method according to claim 14 wherein the product is labeled with a label moiety.

16. The method according to claim 15 wherein the label moiety is an antibody.

17. The method according to claim 14 wherein the capture moiety is an antibody or an antigen-binding fragment thereof.

18. The method according to claim 17 wherein the antibody is bispecific.

19. The method according to claim 14 wherein the coupling is through a lipid anchor attached to the capture moiety optionally through a linker moiety.

20. The method according to claim 14 wherein the coupling is through an antibody or an antigen-binding fragment thereof attached to the capture moiety optionally through a linker.

21. The method according to claim 18 wherein the coupling is through specific binding of the antibody to the cell.

22. A composition obtained from the method according to claim 21.

23. The composition according to claim 21 wherein the capture moiety is an antibody or an antigen-binding fragment thereof.

24. The composition according to claim 23 wherein the antibody is bispecific.

25. The composition according to claim 22 wherein the coupling is through a lipid anchor moiety attached to the capture moiety optionally through a linking moiety.

27. The composition according to claim 22 wherein the coupling is through an antibody or an antigen-binding fragment thereof attached to the capture moiety, optionally through a linker.

28. The composition according to claim 25 wherein the coupling is through specific binding of the antibody to the cell.

29. Cells and progeny thereof separated according to the method of claim 1.

30. Cells separated according to the method of claim 1.

31. A method of analyzing a population of cells to identify or enumerate antigen-specific T cells that secrete and release an amount of product relative to other cells in the population, wherein the product is secreted in response to antigen stimulation, the method comprising the steps of:

labeling the cells by the method according to claim 14,

labeling the cells with at least one additional label that does not label the captured product, and

detecting the amount of product label relative to the additional label.

32. A method of determining a distribution of secretory activity in a cell population enriched in T cells, the method comprising the steps of:

labeling cells by the method according to claim 14, and

determining the amount of product label per cell, wherein the product is secreted and released in response to antigen stimulation.

33. The method according to claim 14 further comprising the steps of:

determining the amount and type of product label per cell wherein distribution of secreted product type and secretory activity for each secreted product type in a population of cells is determined.

34. A method for identifying antigen-specific T cells secreting and releasing at least one product in response to antigen stimulation, comprising the steps of:

combining a mixed population of cells enriched for T cells with at least one first, bispecific, antibody, each antibody, having combining sites specific for a cell surface molecule and at least one product;

exposing the cell population to at least one antigen under conditions effective to elicit antigen-specific stimulation of at least one T cell;

incubating the combination under conditions and for a time sufficient to allow the cells to secrete the at least one product;

adding at least one label moiety; and

detecting the at least one label moiety.

35. The method according to claim 34 further comprising the step of separating the cells secreting the product from the mixed cell population.

36. The method according to claim 34 wherein the cell surface molecule is a naturally occurring cell surface protein.

37. The method according to claim 36 wherein the protein is a cell surface marker.

38. The method according to claim 37 wherein the cell surface molecule is selected from the group consisting of CD2, CD3, CD4, CD5, CD8, CD11b, CD26, CD27, CD28, CD29, CD30, CD31, CD38, CD40L, CD45RO, CD45RA, LAG3, T1/ST2, SLAM, Class I MHC molecules, Class II MHC molecules, T cell antigen receptor, and β_2-microglobulin.

39. The method according to claim 34 wherein the incubation conditions include a high viscosity or gel forming medium.

40. The method according to claim 34 wherein the label moiety is an antibody.

41. The method according to claim 40 wherein the antibody comprises a detectable label.

42. The method according to claim 41 wherein the label is selected from the group consisting of fluorophores, radioactive isotopes, chromophores and magnetic particles.

43. The method according to claim 40 wherein the label moiety is detected by fluorescence activated cell sorting.

44. The method according to claim 43 wherein the label moiety is detected by a third antibody.

45. The method according to claim 44 wherein the label moiety is coupled to digoxigenin and the third antibody is specific for digoxigenin.

46. The method according to claim 45 wherein the third antibody comprises a detectable label.

47. The method according to claim 46 wherein the label is selected from the group consisting of fluorophores, radioactive isotopes, chromophores, and magnetic particles.

48. The method according to claim 47 wherein the label moiety is detected by fluorescence activated cell sorting.

49. The method according to claim 34 wherein the label moiety comprises a magnetizable moiety.

50. The method according to claim 49 wherein the label moiety is detected by a third antibody coupled to a magnetizable moiety.

51. A method of treating a disease or condition related to a population of antigen-specific T cells comprising administering to an individual in need thereof an amount of a cell population enriched in antigen-specific T cells effective to treat the condition.

52. The method according to claim 51, wherein the condition is selected from the group consisting of an autoimmune disorder, graft rejection, and an allergic response.

53. The method according to claim 51, wherein the condition is a result of a lack of adequate control of the condition by antigen-specific T cells.

54. The method according to claim 53, wherein the condition is cancer.

55. The method according to claim 53, wherein the condition is an infection.

56. A kit for use in the detection of antigen-specific T cells that secrete a product in response to antigen stimulation, the kit comprising:

a product capture system comprised of at least one anchor moiety and at least one capture moiety; and

at least one label moiety.

57. The kit according to claim 56, wherein the capture moiety comprises at least one bispecific antibody having at least one antigen recognition site for at least one cell type and at least one antigen recognition site specific for the product.

58. The kit according to claim 57 wherein the at least one bispecific antibody and the at least one label moiety are in a single vial.

59. The kit according to claim 57 wherein the at least one bispecific antibody binds to the cell through a cell surface molecule.

60. The kit according to claim 57 wherein the cell surface molecule is a naturally occurring cell surface protein.

61. The kit according to claim 57 wherein the cell surface molecule is a cell surface marker.

62. The kit according to claim 61 wherein the cell surface molecule is selected from the group consisting of CD2, CD3, CD4, CD5, CD8, CD11b, CD26, CD27, CD28, CD29, CD30, CD31, CD38, CD40L, CD45RO, CD45RA, LAG3, T1/ST2, SLAM, Class I MHC molecules, Class II MHC molecules, T cell antigen receptor, and β_2-microglobulin.

63. The kit according to claim 55 wherein the incubation conditions include a high viscosity or gel forming medium.

64. The kit according to claim 63 wherein the medium is selected from the group consisting of gelatin, agarose, alginate and combination thereof,

65. The kit according to claim 57 wherein the label moiety is an antibody.

66. The kit according to claim 65 wherein the antibody comprises a detectable label.

67. The kit according to claim 66 wherein the detectable label is selected from the group consisting of fluorophores, radioactive isotopes, chromophores, and magnetic particles.

68. The kit according to claim 67 wherein the label moiety is detected by fluorescence activated cell sorting.

69. The kit according to claim 65 wherein the label moiety is detected by a third antibody.

70. The kit according to claim 69 wherein the label moiety is coupled to digoxigenin and the third antibody is specific for digoxigenin.

71. The kit according to claim 69 wherein the third antibody comprises a detectable label.

72. The kit according to claim 65 further comprising a biological modifier.

73. The kit according to claim 56 further comprising a cell-cell cross-contamination reducing capture system.

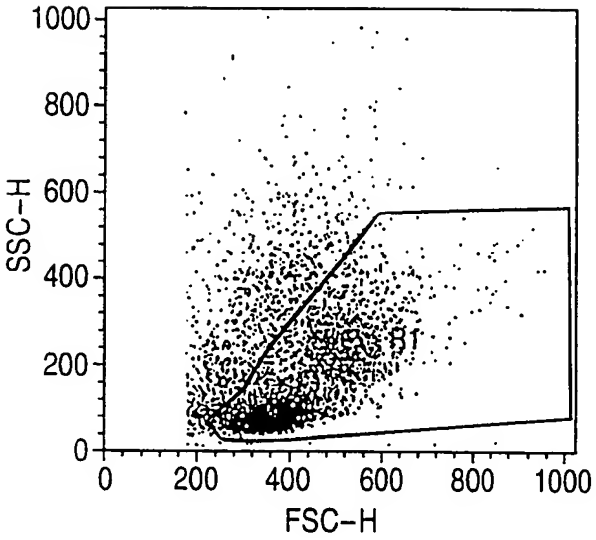


FIG. 1A

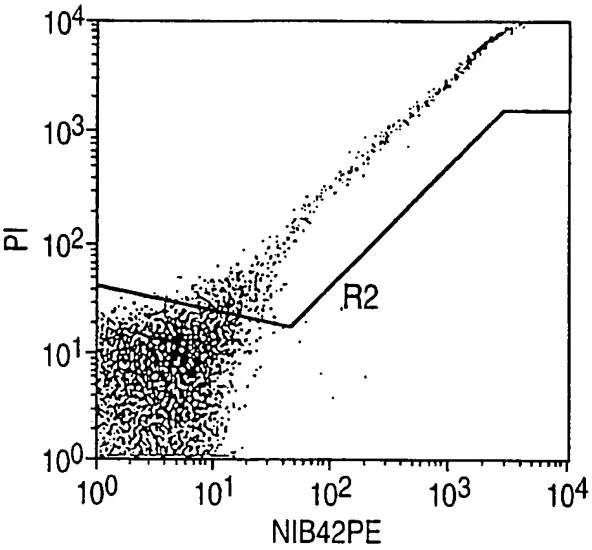


FIG. 1B

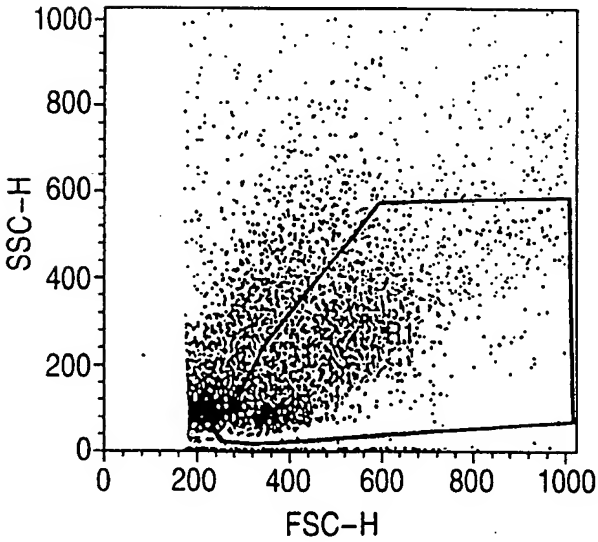


FIG. 1C

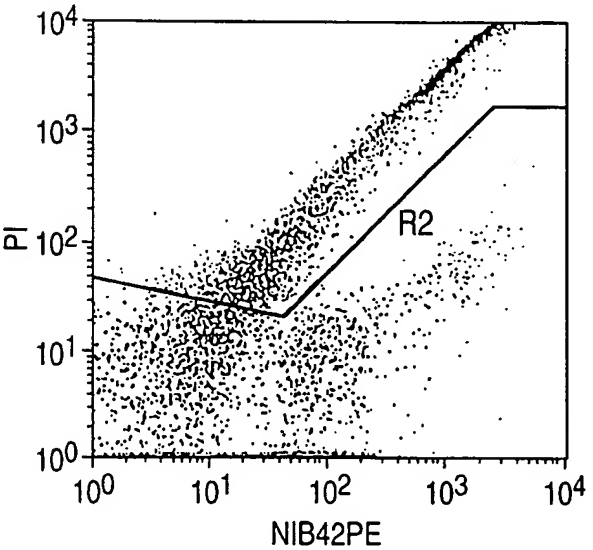


FIG. 1D

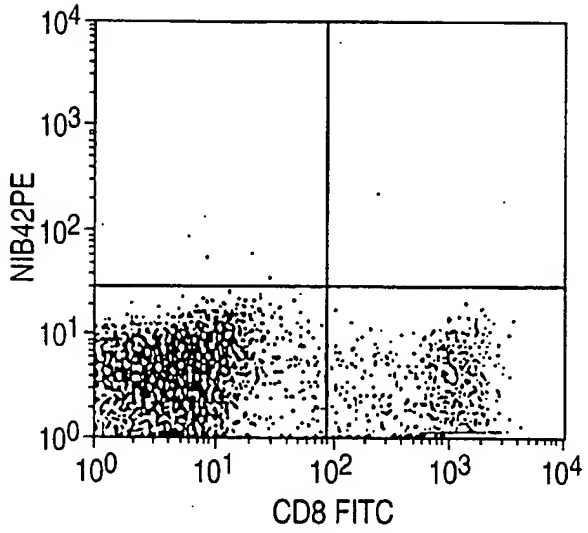


FIG. 1E

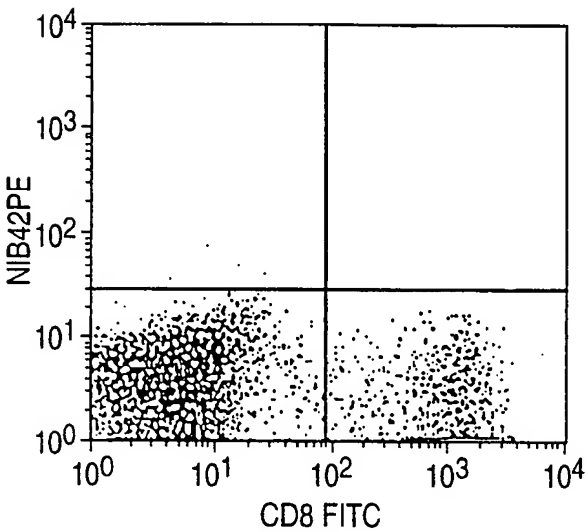


FIG. 1F

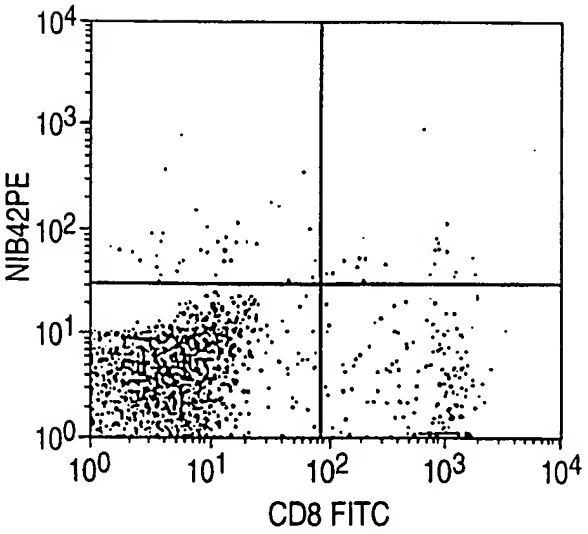


FIG. 1G

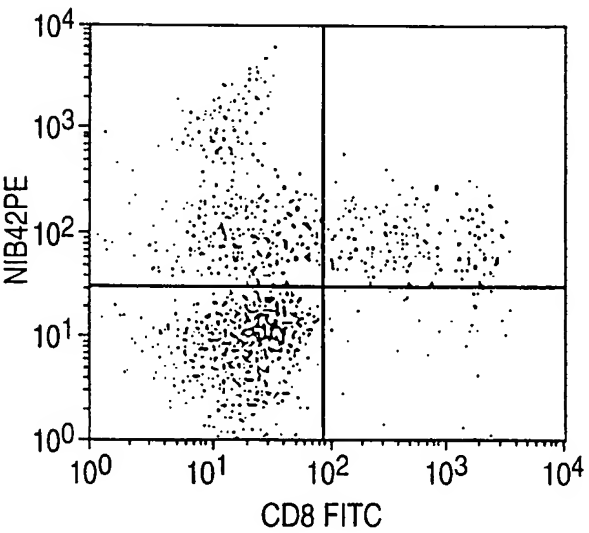


FIG. 1H

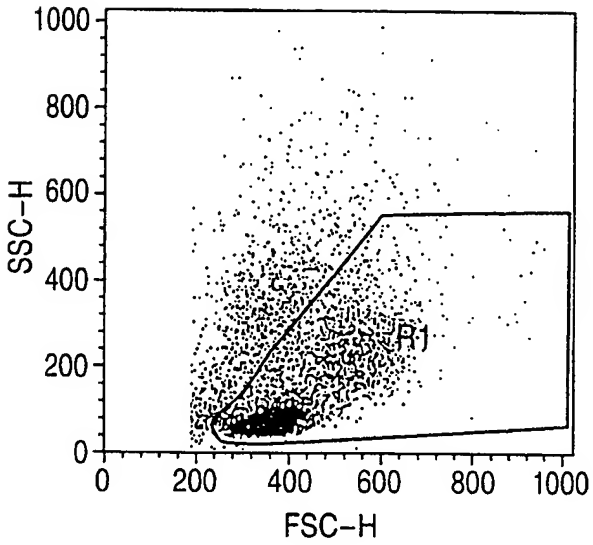


FIG. 1I

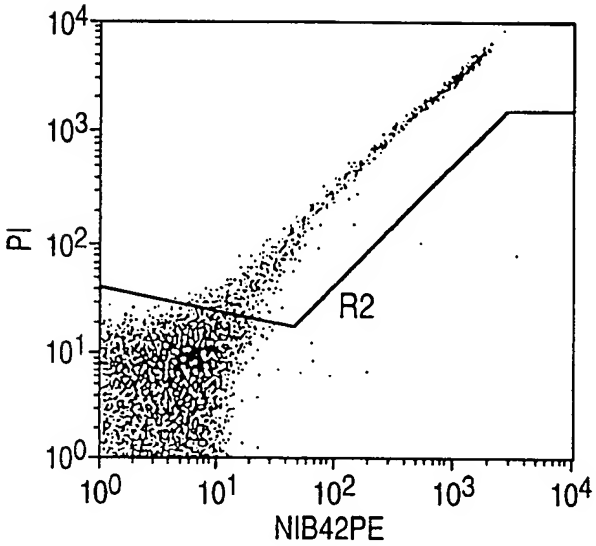


FIG. 1J

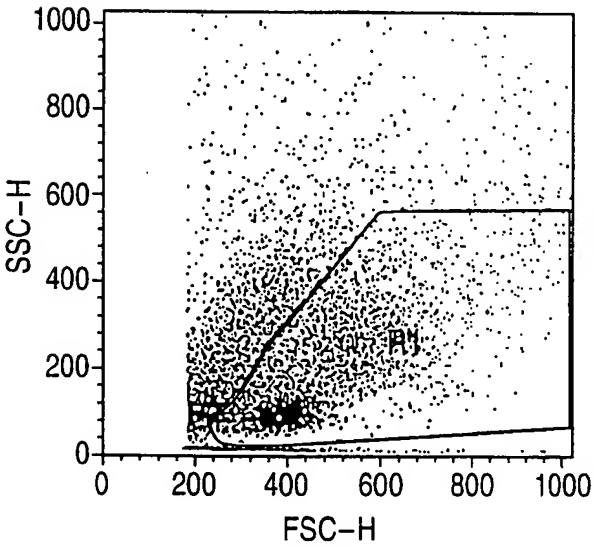


FIG. 1K

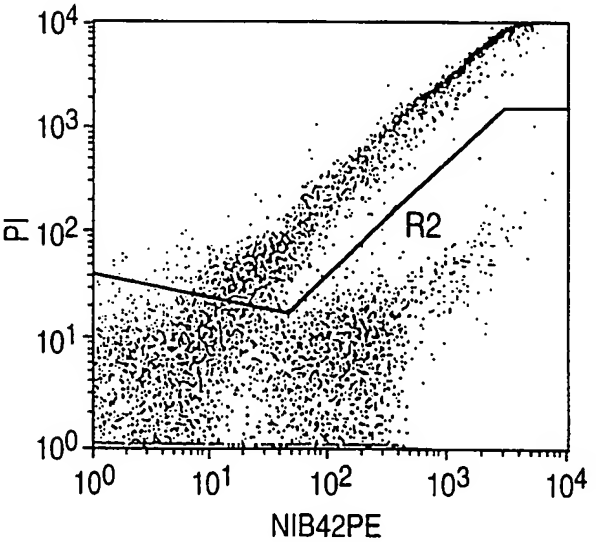


FIG. 1L

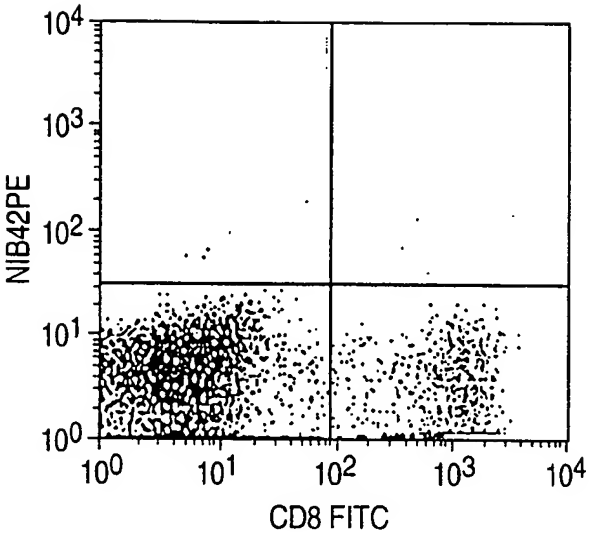


FIG. 1M

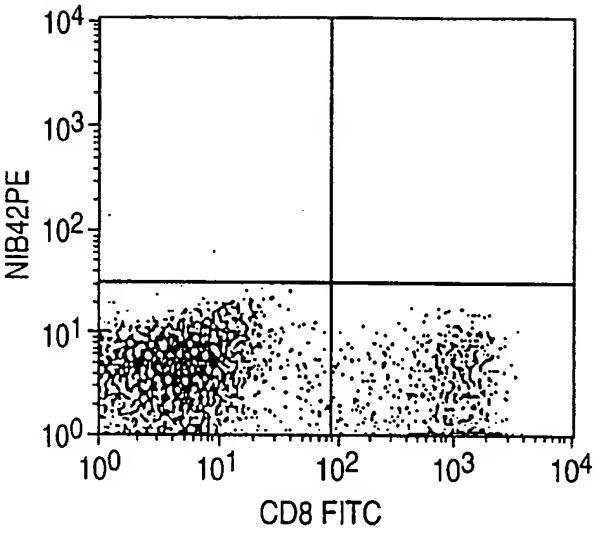


FIG. 1N

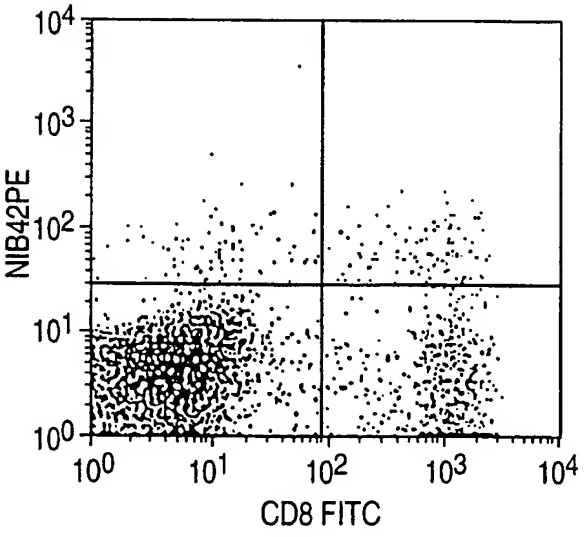


FIG. 1O

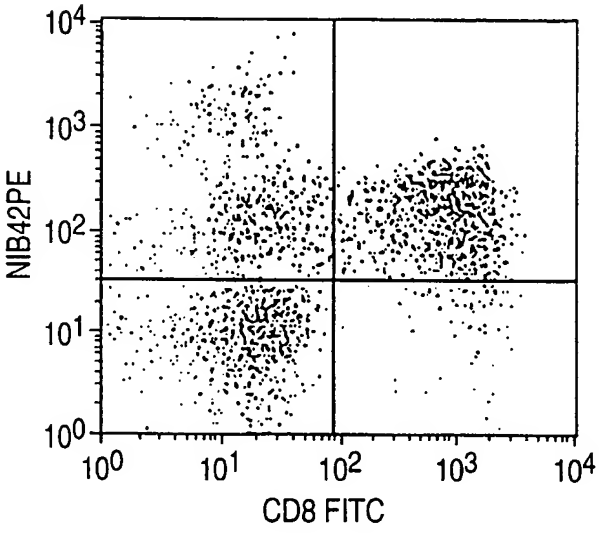


FIG. 1P

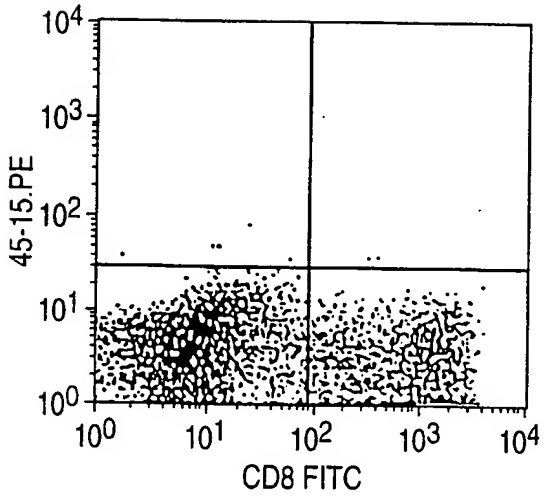


FIG. 2A

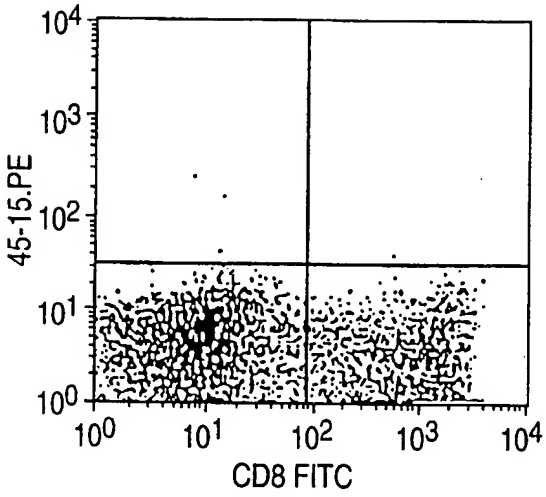


FIG. 2B

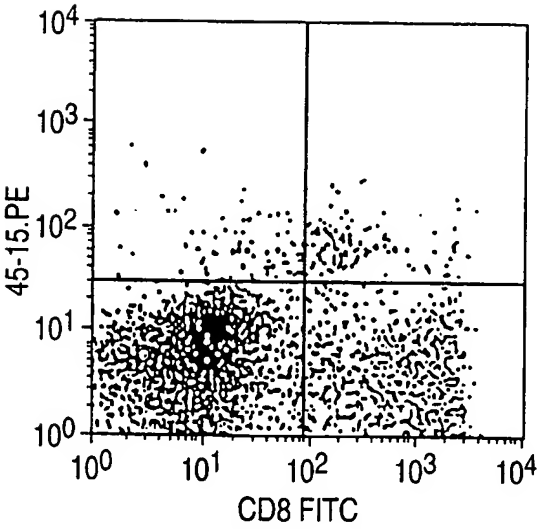


FIG. 2C

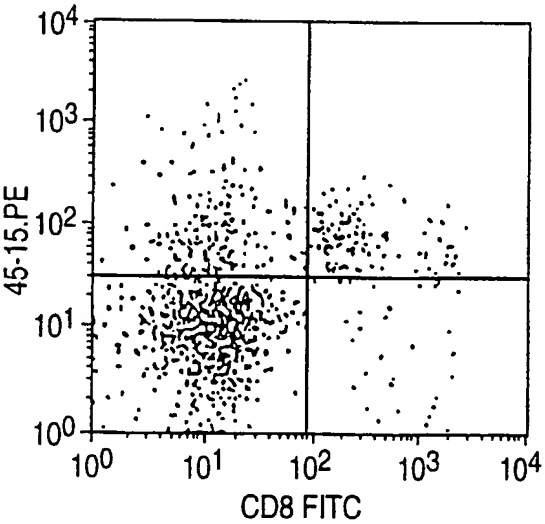


FIG. 2D

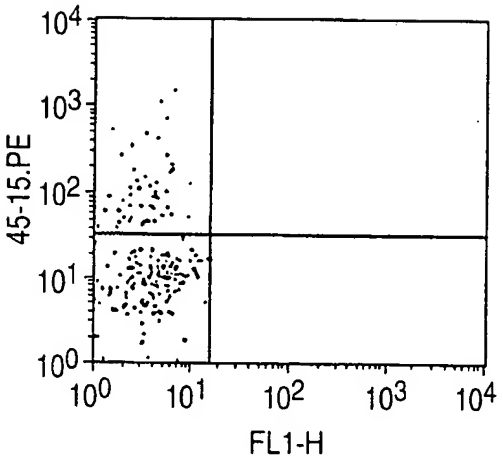


FIG. 2E

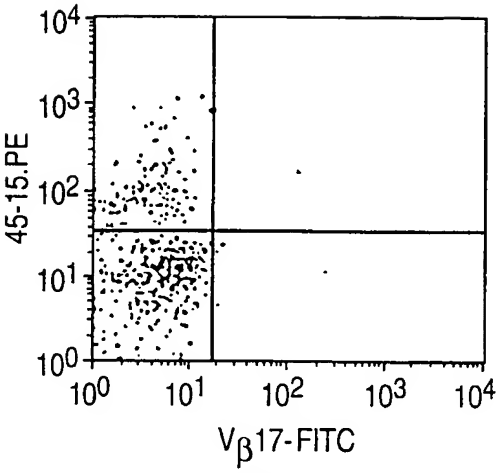


FIG. 2F

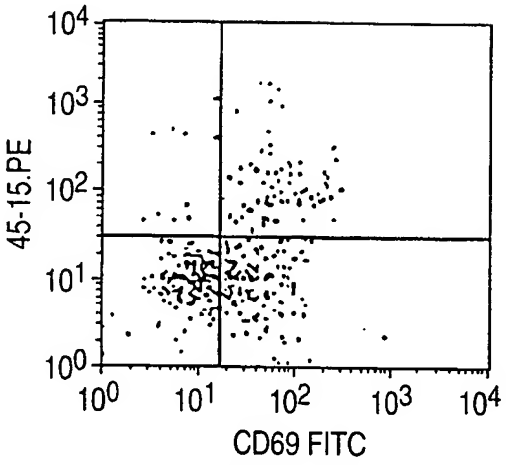


FIG. 2G

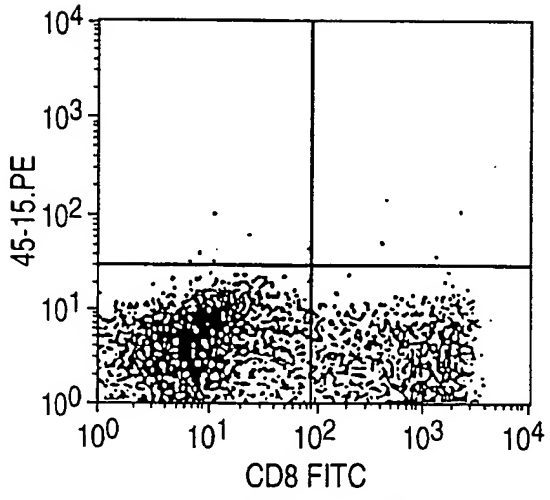


FIG. 2H

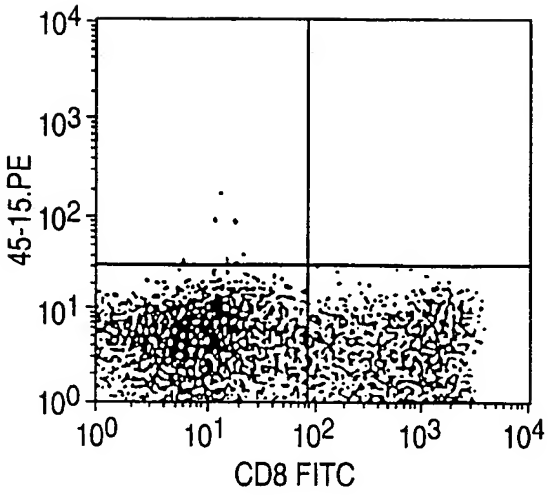


FIG. 2I

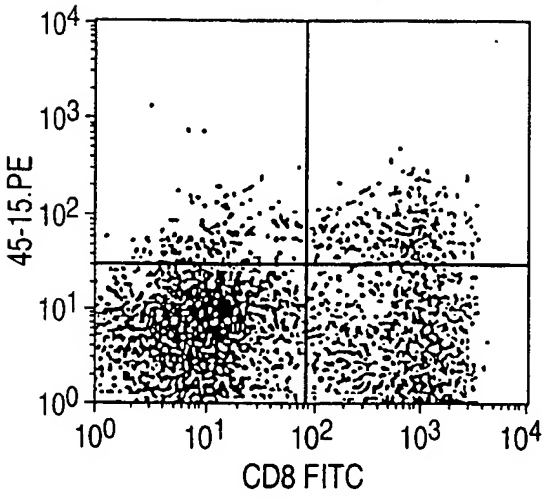


FIG. 2J

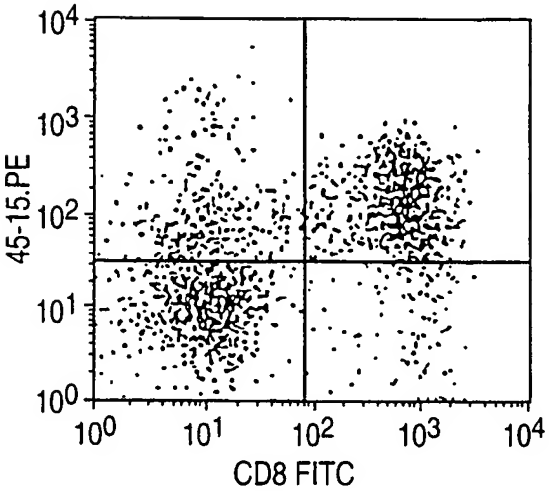


FIG. 2K

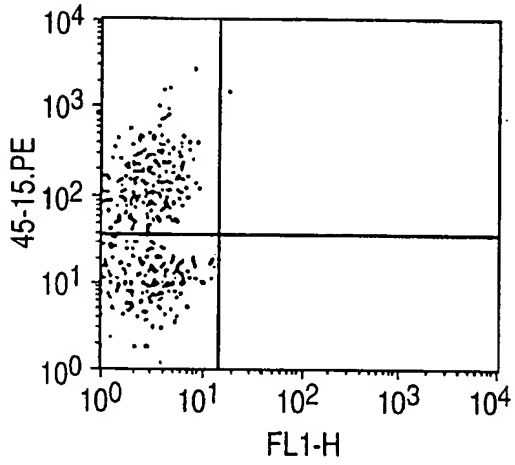


FIG. 2L

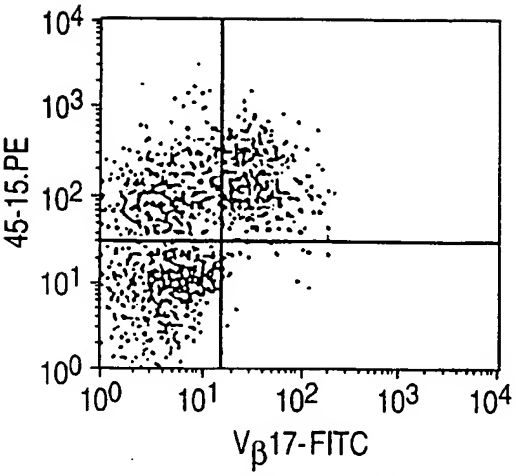


FIG. 2M

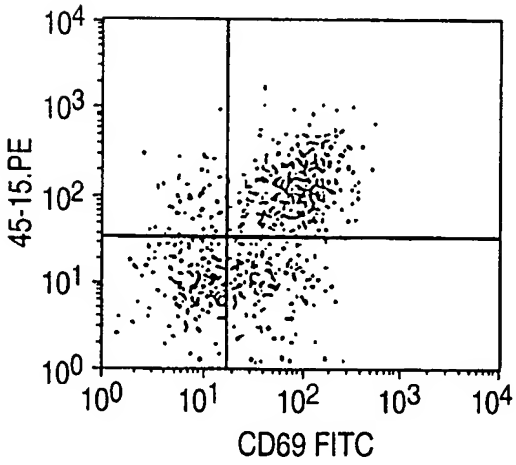


FIG. 2N

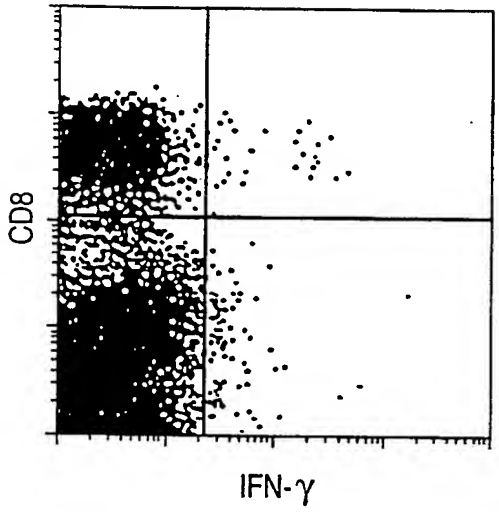


FIG. 3A

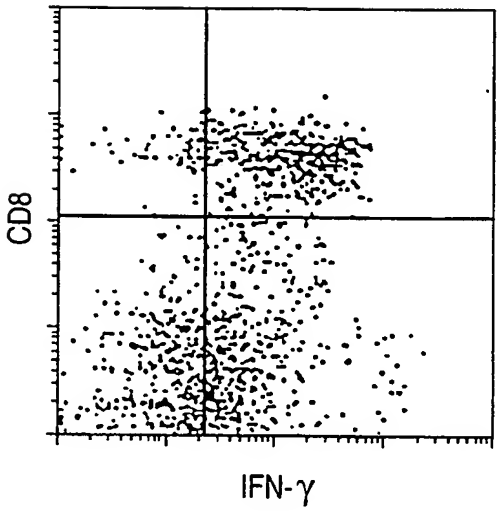


FIG. 3B

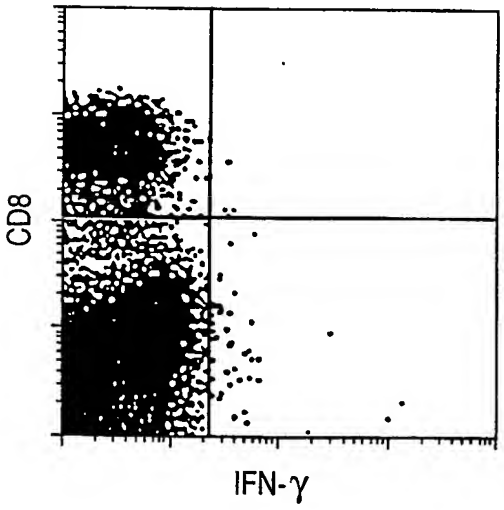


FIG. 3C

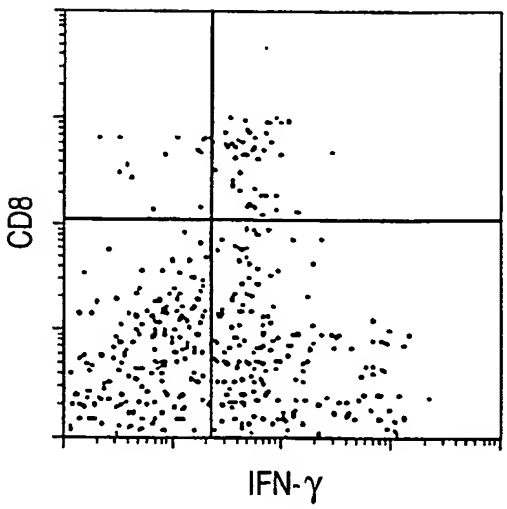


FIG. 3D

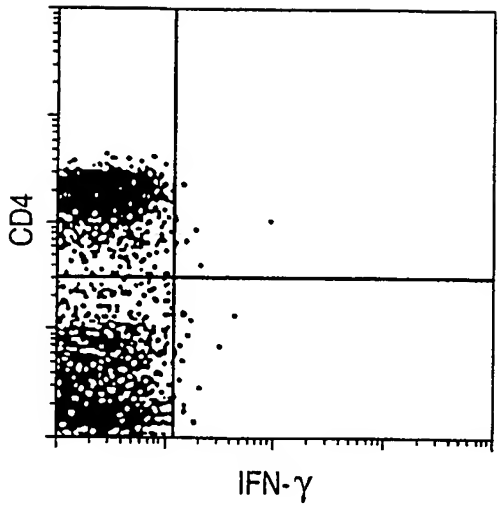


FIG. 3E

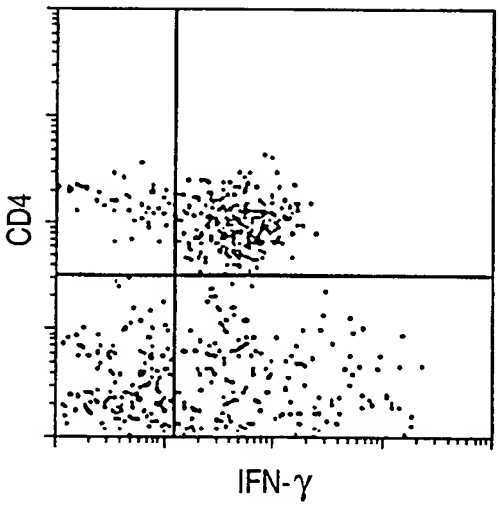


FIG. 3F

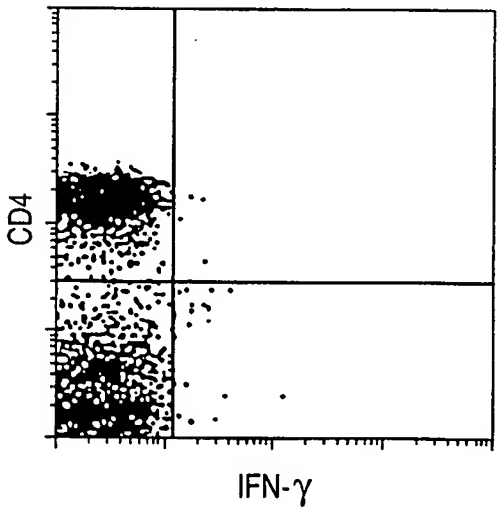


FIG. 3G

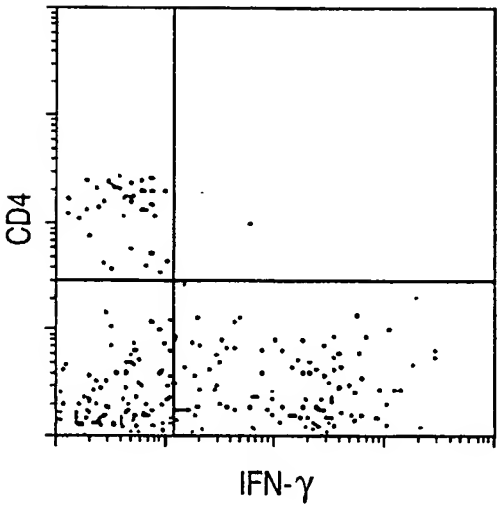


FIG. 3H

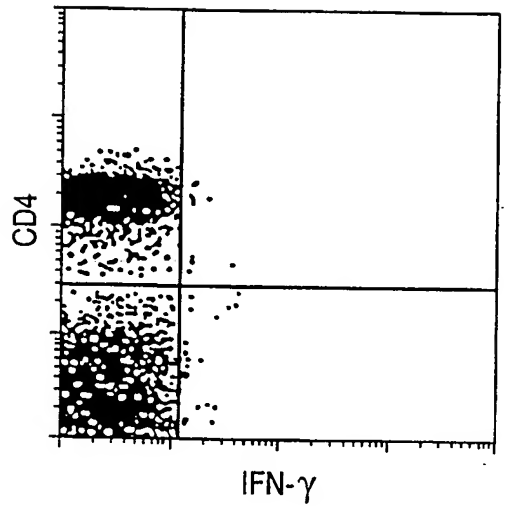


FIG. 3I

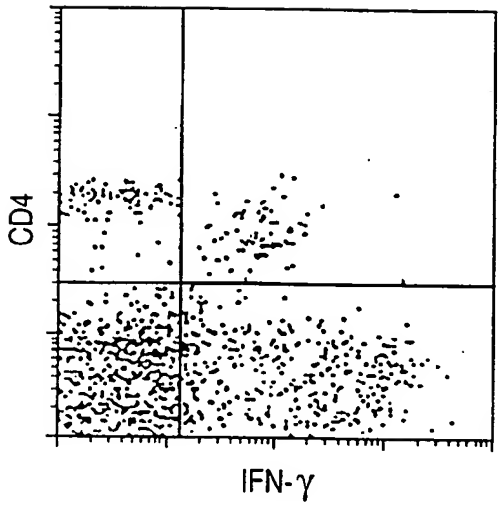


FIG. 3J

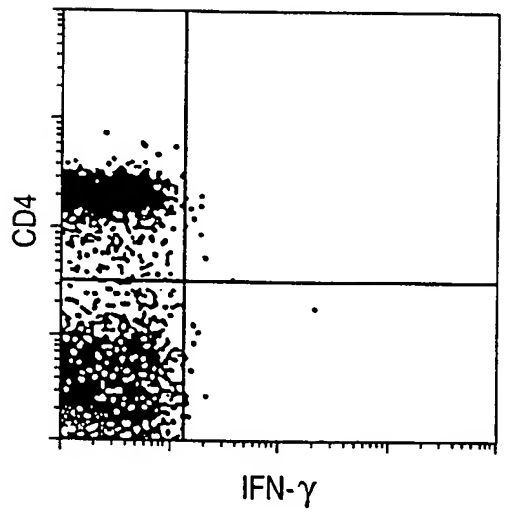


FIG. 3K

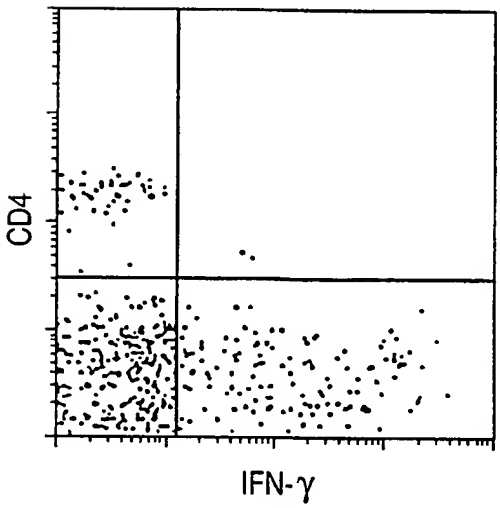


FIG. 3L

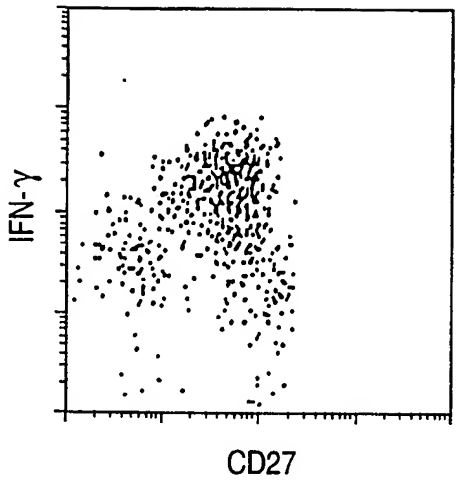


FIG. 4A

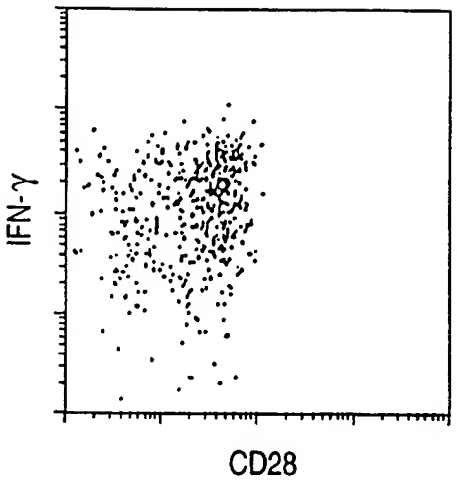


FIG. 4B

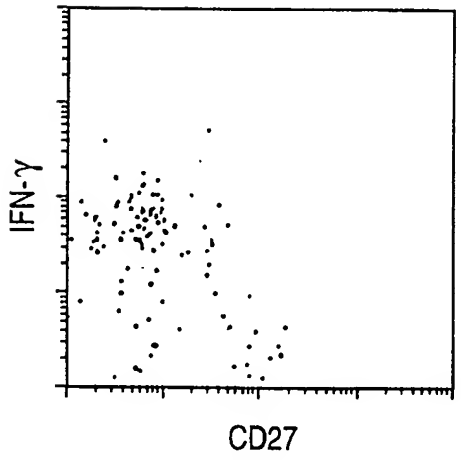


FIG. 4C

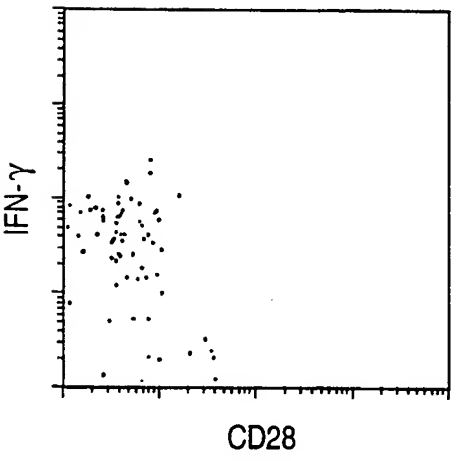
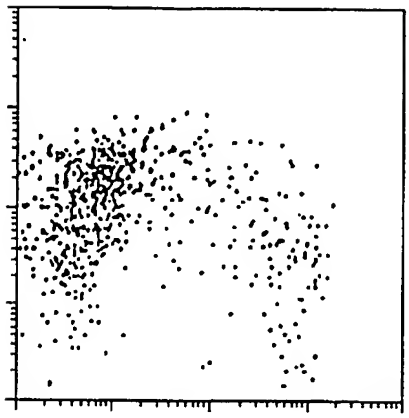
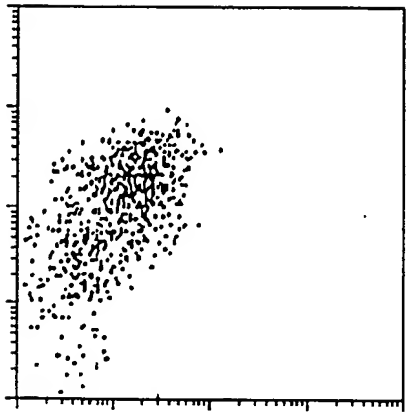


FIG. 4D



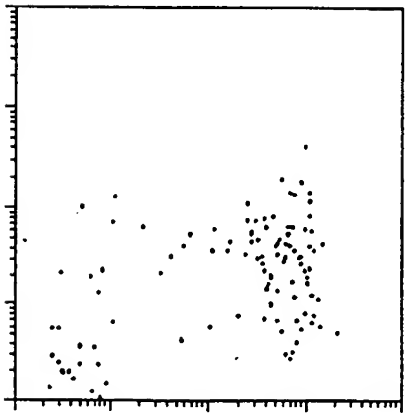
CD57

FIG. 4E



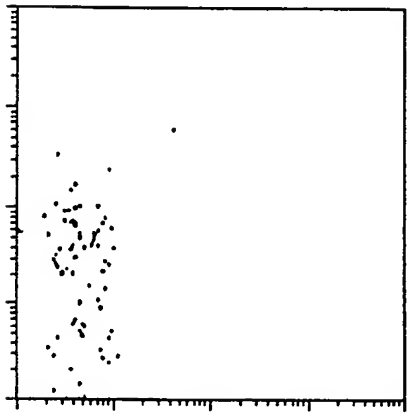
$V_\beta 17$

FIG. 4F



CD57

FIG. 4G



$V_\beta 17$

FIG. 4H

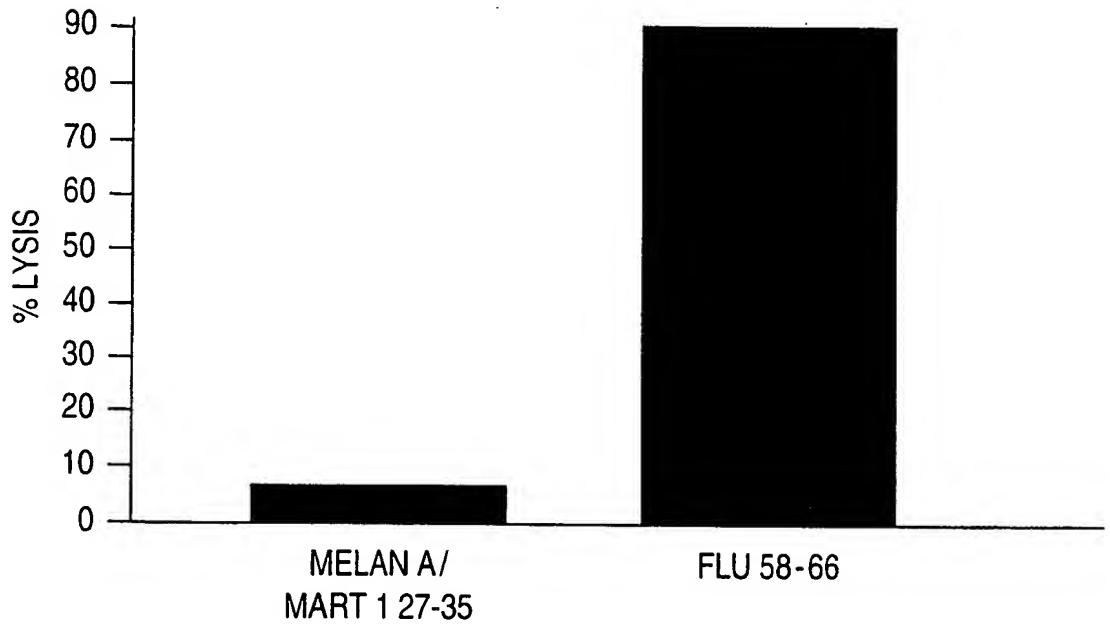


FIG. 5

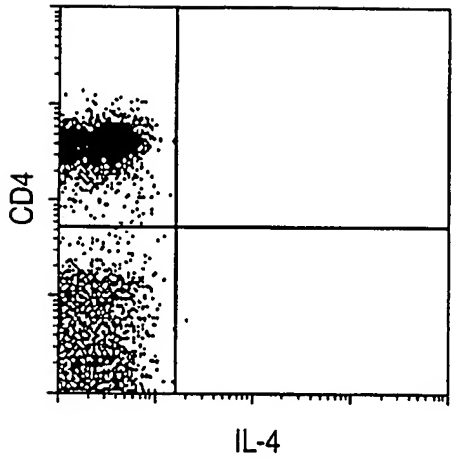


FIG. 6A

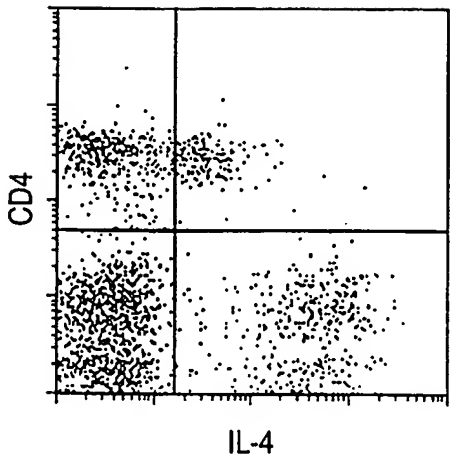


FIG. 6B

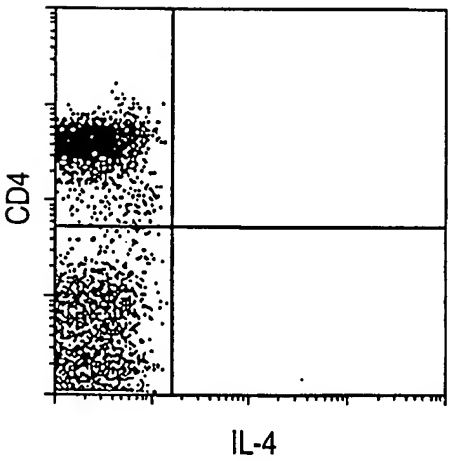


FIG. 6C

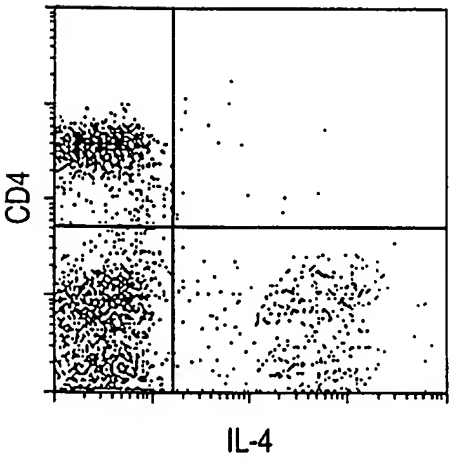


FIG. 6D

INTERNATIONAL SEARCH REPORT

Inter 'onal Application No
PCT/US 99/10200

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/569 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 09117 A (MILTENYI BIOTEC INC ;MILTENYI STEFAN (DE); RADBRUCH ANDREAS (DE);) 28 April 1994 (1994-04-28) cited in the application the whole document ---	1-70
X	MANZ ET AL: "Analysis and sorting of live cells according to secreted molecules located to a cell surface affinity matrix" PROC.NATL.ACAD.SCI. USA, vol. 92, March 1995 (1995-03), pages 1921-1925, XP002119208 the whole document --- -/--	1-70

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 October 1999	04/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Routledge, B

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 99/10200

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	ASSENMACHER M ET AL: "FLUORESCENCE-ACTIVATED CYTOMETRY CELL SORTING BASED ON IMMUNOLOGICAL RECOGNITION" CLINICAL BIOCHEMISTRY, vol. 28, no. 1, 1 February 1995 (1995-02-01), page 39/40 XP002046269 ISSN: 0009-9120 page 40, left-hand column, paragraph 3 -right-hand column, paragraph 1 ---	1-70
A	US 5 750 356 A (SPACK EDWARD G ET AL) 12 May 1998 (1998-05-12) claims -----	1-70

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

national application No.
PCT/US 99/10200

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 51-55 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter 'onal Application No
PCT/US 99/10200

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9409117 A	28-04-1994	AU 679949 B	17-07-1997
		AU 5538594 A	09-05-1994
		CA 2146974 A	28-04-1994
		EP 0667896 A	23-08-1995
		JP 8504574 T	21-05-1996
US 5750356 A	12-05-1998	AU 3138797 A	05-01-1998
		EP 0912895 A	06-05-1999
		WO 9745735 A	04-12-1997